Defining physiological normoxia for improved translation of cell physiology to animal models and man

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Short title: Redefining hyperoxia, normoxia and hypoxia *in vitro*

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Abstract

The extensive oxygen gradient between the air we breathe (PO_2 ~21 kPa) and its ultimate distribution within mitochondria (as low as ~0.5-1 kPa) is testament to the efforts expended in limiting its inherent toxicity. It has long been recognised that cell culture undertaken under room air conditions falls short of replicating this protection *in vitro*. Despite this, difficulty in accurately determining the appropriate O_2 level(s) in which to culture cells, coupled with a lack of the technology to replicate and maintain a physiological O_2 environment *in vitro*, has hindered addressing this issue thus far. In this review, we aim to address the current understanding of tissue PO_2 distribution *in vivo*, and summarize the attempts made to replicate these conditions *in vitro*. The state-of-the-art techniques employed to accurately determine O_2 levels, as well as the issues associated with reproducing physiological O_2 levels *in vitro*, are also critically reviewed. We aim to provide the framework for researchers to undertake cell culture under O_2 levels relevant to specific tissues and organs. We envisage that this review will facilitate a paradigm shift, enabling translation of findings under physiological conditions *in vitro* to disease pathology and the design of novel therapeutics.

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I. Introduction

Although most organisms on Earth possess a carbon-based skeleton, oxygen fuels life. The conversion of a CO_2-rich atmosphere to one containing precious O_2, initially by photosynthesising cyanobacteria and later by plants, was the pivotal step in the evolution of multicellular life on Earth (324). However, O_2 is an inherently dangerous molecule, and hence its utilization as a metabolic substrate is closely associated with the development of highly efficient detection (e.g. hypoxia inducible factors (516, 630)), defense (glutathione (195, 387) and antioxidant enzymes (261)) systems. Despite this, the damage caused by a lifetime breathing O_2 may ultimately affect lifespan (222, 324). Several excellent and comprehensive reviews have discussed the various roles played by O_2 in mammalian physiology (59, 119, 333, 565, 594, 679, 699), and thus these will not form the primary focus of this review article. Nevertheless, a brief overview is important to inform the themes addressed herein.

The principle role of O_2 in mammalian physiology is as the terminal electron acceptor in the electron transport chain. In this capacity, a single oxygen atom is reduced to H_2O in the presence of 2 protons and 2 electrons. Such reductive/oxidative (redox) reactions are fundamental in O_2 physiology and are utilized by cytosolic enzymatic systems in addition to mitochondria. Aside from molecular O_2, oxygen strongly influences cellular physiology through the formation of reactive oxygen species (ROS). First considered merely as unwanted by-products of oxidative phosphorylation and dysfunctional enzymatic reactions (222), reactive oxygen species are now considered to play functional and non-redundant roles in cell signaling in physiology as reviewed previously (119).

Our increased understanding of oxygen's role in human physiology is paralleled by the growing number of primary research publications (see Figure 1). To date, over half a million research articles reference oxygen as a keyword, and the number per year has steadily increased over the last century. Attention has shifted from initial interests in the mechanisms of cellular O_2 utilization to consequences associated with dysfunction in these processes leading ultimately to the production of reactive oxygen species (Figure 1). Another key theme to have emerged is the concept of hypoxia, a pathological reduction in O_2 availability now accepted as a hallmark of disease conditions (522). Indeed, a significant proportion of O_2-related research still focuses on the consequences of O_2 deprivation (Figure 1). In view of the importance of maintaining O_2 levels within a finite range at the whole organism level, we here *readdress* the role of O_2 in mammalian cell culture.

The invention of an O_2 sensitive electrodes (87), and in recent years phosphorescent dyes (116, 610), has enabled researchers to resolve tissue O_2 distribution *in vivo*. Whilst incredibly heterogeneous, at no point beyond the mouth is PO_2 equivalent to room air (20.9 kPa). Despite

this, the culture of mammalian cells is routinely conducted under atmospheric O_2, conditions hyperoxic with respect to the tissue of origin. Although this discrepancy has been widely acknowledged (215, 455, 633), only in recent years have primary research publications addressed this issue directly (Figure 1 inset). A consensus approach to tackling this concept is still lacking, largely plagued by incorrect nomenclature and widely differing experimental conditions. This review aims to provide an insight into the current knowledge regarding tissue O_2 distribution *in vivo*, and in doing so to redefine the term normoxia for more appropriate use in *in vitro* experiments. Techniques used to monitor O_2 levels both *in vivo* and *in vitro* will be described initially, followed by an overview of experimental issues associated with varying O_2 levels *in vitro*. Physiological O_2 levels in numerous tissues and the existing literature concerning replication of these conditions *in vitro* are summarised and critically discussed. The development of cell culture techniques revolutionized medical science in the 1950s, yet maintenance of cultured cells or tissues under non-physiological O_2 levels limits translation from bench to beside. In many fields, cell culture under atmospheric, room air O_2 levels has been assumed to reflect 'normoxia' largely due to the complexity of conducting experiments in an oxygen-regulated workstation. This review aims to readdress the conditions in which all mammalian cells should be cultured with the aim of more effective translation of *in vitro* science into potentially life-saving therapies.

II. Techniques to measure oxygen in cells and tissues

Necessity often dictates that improvements in experimental techniques frequently precede advances in scientific knowledge, and such is the case with oxygen related physiology (Figure 1). The study of the physiology and biochemistry of molecular O_2 was aided immensely in the 1950's by the development of O_2-sensitive electrodes, which allowed the direct quantification of O_2 levels in biological fluids (87). A number of innovative techniques have since been developed, facilitating more advanced experimental measurements with greater spatial and temporal resolution. Wire electrodes can offer excellent spatial definition over a very small sample area, whereas phosphorescence imaging techniques can offer greater stability over time and over a greater sampling size. Notably, these techniques rarely monitor O_2 concentration directly but rather PO_2, which is directly proportional to concentration, or hemoglobin saturation. The following section outlines the basic principles of the most well established techniques currently used to monitor cellular and tissue PO_2, providing a basis for discussion of data obtained using such equipment in later sections.

A. Oxygen sensitive electrodes

The chemical reduction of molecular O_2 on a polarized (usually 0.6-7V) platinum cathode generates a current proportional to the concentration of O_2 present. This concept led to the development of O_2 sensitive electrodes in the early 1950's, consisting of a polarised platinum cathode and non-polarizable silver anode (see Figure 2A). Although effective in determining

dissolved O_2, these early electrodes were not suitable for measuring tissue or blood O_2 due to the accumulation of cells/proteins on the cathode limiting reactivity. This problem was solved by Leland Clark and colleagues who, in 1953, described the fabrication of a platinum electrode with an O_2-permeable cellophane coating (87)(Figure 2Ai). The addition of a selectively permeable membrane permitted the measurement of PO_2, as O_2 must now diffuse across its partial pressure gradient to react on the cathode. These electrodes, commonly referred to now as 'Clark Electrodes', were greatly utilized for the measurement of liquid PO_2 for blood gas analysis and early mitochondrial respiration experiments (73), yet their relatively large size (<1mm) prevented accurate measurements of tissue PO_2. Efforts during the 1960's culminated in the introduction of the 'Whalen' type electrode; a recessed-tip gold alloy cathode with a diameter of 1-3µm (644)(Figure 2Aii). By attaching an O_2-electrode to a micromanipulator, tissue PO_2 histograms were easily obtainable and rapidly became the preferred method for reporting PO_2 (see Section VI). In recent decades, the use of electrodes has decreased in favor of newer techniques (discussed below) due to the following disadvantages: (i) by virtue of their chemistry, electrodes consume O_2 (proportional to their diameter) and therefore may be considered less reliable especially when attempting to measure very low levels of O_2, (ii) the small sampling area means measuring large areas of tissue is tedious, and (iii) many have shown that tissue penetration by electrodes produces artefactual changes in tissue PO_2 resulting from blood vessel rupture or cell disruption (266). The second point has been partially addressed through the development of the 'multiwire' platinum electrode (296), in which eight independent Clark type electrodes are housed within a hemispherical structure of roughly ~50µm diameter. Whilst greatly increasing the sampling area, these electrodes are confined to surface PO_2 measurements and hence their use is limited.

B. Hemo/myoglobin spectroscopy

Prior to the invention of O_2-sensitive electrodes, the only readout of blood oxygenation available was the spectrophotometric analysis of hemoglobin (Hb), although this was only employed clinically in the late 1970's following the development of the pulse oximeter by Aoyagi and colleagues (13). This technique relies on the change in absorbance spectrum of hemoglobin upon binding to O_2 (see Figure 2B), characterised by a reduction in absorbance in the red region (650-700nm) and an increase in the near-infrared (900-1000nm) region. Isolation of the hemoglobin-derived signal from intrinsic signals of the surrounding tissues and venous blood is achieved by only measuring the pulsatile changes in absorbance. So called Pulse oximeters are extensively used clinically to monitor peripheral arterial blood O_2 saturation, which is 95-99% in normoxic patients. In the laboratory, a technique to measure Hb saturation in the microvasculature was developed by Pittman and Duling in 1975 (460, 461), in which the effects of light scattering by red blood cells was accounted for. A similar technique has also been used to measure myoglobin saturation in cardiac and skeletal muscle (89, 252). In the context of defining tissue PO_2, Hb saturation can be converted into approximate PO_2 based on the known Hb saturation curves for

the appropriate species (594), although this conversion is subject to changes in local pH/PCO_2 and temperature and hence should be used with caution.

C. Fluorescent and phosphorescent probes

Optical techniques to monitor cellular and tissue PO_2 were developed by David Wilson and colleagues in the late 1980's, who introduced platinum (Pt^{II}) and palladium (Pd^{II})-porphyrin based probes which exhibit quenched phosphorescence in the presence of molecular O_2 (497, 610). These have been complemented by similar complexes utilizing ruthenium (Ru^{II}) (348, 366, 580) or iridium (Ir^{III}) (108), all of which can be conjugated to various macromolecular carriers or polymers to improve solubility or phosphorescence yield. A complete description of O_2-sensitive lumiphores can be found elsewhere (115). In principle, complexes are excitable in the UV range (350-410 nm) and emit in the far red (630-700 nm) region, allowing excellent resolution in biological media, with decay lifetimes in the µs range. Typically, measurements are taken using time-resolved phosphorescence, whereby the probe is excited and the O_2-sensitive decay in resultant emission intensity resolved over time (see Figure 2C). First generation phosphorescent complexes were hydrophilic and hence measurements were confined to the vasculature (535, 583), but subsequent variations have generated hydrophobic equivalents that can penetrate the cell membrane, such as Oxyphor G4 (136) or MitoXpress® INTRA (151). The addition of other chemical moieties to the basic metal-porphyrin structure has also alleviated issues with non-specific protein binding that altered the spectral properties of first generation compounds *in* vivo (115). With these, simultaneous measurements of blood and tissue PO_2 have been reported (9), as well as intracellular PO_2 within monolayers in culture (75, 151, 615). Ru^{II} complexes have largely been utilized in the OxyLite™ probe, where they are immobilized in a sensor tip (~200µm in diameter) and excited via a fibre-optic cable, functioning in an analogous manner to a traditional electrode.

D. Magnetic, paramagnetic and electron spin resonance

Positron emission tomography (PET), magnetic resonance imaging (MRI) and electron spin resonance (ESR) techniques have been developed to image regions of hypoxia *in vivo*, as reviewed elsewhere (60). Imaging using PET employs traditional biochemical markers of tissue hypoxia, such as the imidazole compounds (see below), containing the radionucleotide ^{18}F as a short-lived source of protons. These compounds accumulate within regions of hypoxia and have been useful in imaging tumor PO_2 in animal models and in the clinical setting (356). More recently, ^{64}Cu containing bisthiosemicarbozones have been targeted as potentially useful markers of hypoxia, with ^{64}Cu-ATSM showing high selectivity for regions of relative hypoxia in the ischemic Langendorff heart (169) and atherosclerotic lesions in rodents (426), as well as tumors in man (602). An example of ^{64}Cu-ATSM accumulation in the myocardial ischemic core is provided in Figure 2D. MRI oximetry emerged in the late 1980s following the discovery that rates of relaxation to perfluorocarbon (PFC) compounds were linearly related to PO_2 (572). When combined with

traditional ^{1}H MRI anatomical imaging, MRI oximetry can provide useful spatial resolution in both animals and man (680). In the lungs, PO_2 distribution can also be monitored by MRI using inhaled hyperpolarized ^{3}He, the decay of which is increased in the presence of O_2 (127) (see Figure 7). In addition to using exogenous probes, deoxy-hemoglobin is a paramagnetic species itself, and its relative proportion to oxyhemoglobin can be visualized as a disturbance in the transverse relaxation time (T_2*) of the surrounding protons in water. This led to the development of blood-oxygen level dependent MRI imaging (BOLD-MRI) (436, 577) which uses standard MRI protocols to monitor changes in hemoglobin saturation, and is widely used clinically. One major advantage of BOLD-MRI over PFC-MRI and PET is that it does not require the introduction of an exogenous probe, mitigating fears over chemical or radioactive toxicity. Unfortunately, BOLD-MRI is not capable of determining absolute fluid PO_2 and hence cannot be used to monitor tissue PO_2. Molecular O_2 is weakly paramagnetic itself, and can be detected as a small but definitive increase in the longitudinal relaxation time (T_1) of the surrounding protons, first identified using NMR (590) and later confirmed using MRI (267, 432, 656). Notably, changes in T_1 rates occur under conditions in which very limited increases in Hb saturation (and therefore T_2* rates) were detected (656). Whilst this technique has been used to determine absolute increases in O_2 content (ml/L blood) following hyperoxic inspiration, it has had limited application to determine absolute physiological tissue O_2 concentration. Varying T_1 times were reported in the liver, kidney and muscle of rabbits which, when combined with simultaneous measurements of T_2* rates, may be indicative of tissue PO_2 (432, 656).

Much like PET and PFC-MRI, ESR oximetry relies on the interaction between a probe and molecular O_2. The most commonly used probes to measure PO_2 using ESR are lithium based cyanines (LiXC) and india ink (8), which are biocompatible particulate probes and have been used to report tissue PO_2 in liver (275), heart (487), lungs (487), digestive tract (228) and bone (360). The presence of O_2 in close proximity to the ESR probe causes a broadening of the probe spectrum, measurable in the line-width, which is directly proportional to PO_2. Hence, unlike PET and MRI, ESR permits the direct quantification of tissue PO_2, and can be used for long periods of time due to the inert and stable nature of the spin probes (8). Unfortunately, the complex and costly equipment required to detect spin resonance limits the application of ESR to specialized facilities.

E. Immunohistochemical methods

The techniques discussed so far have been developed to monitor relative or absolute tissue PO_2. Complimentary to these has been the immunohistochemical identification of hypoxic areas using exogenous dyes like pimonidazole or EF5 (357, 474) or antibodies raised against known hypoxia-inducible target proteins such as HIF or CA9, which rely on the cellular response to lowered O_2 levels rather than the absolute PO_2. Pimonidazole and EF5 are 2-nitromidazole derivatives

containing a NO_2 group attached to an imidazole ring, which undergoes rapid reduction to produce a highly reactive intermediate species that will react with any macromolecule in the vicinity. At a PO_2 ~>1.5 kPa O_2, this reduction is prevented and thus 2-nitromidazole staining is selective for regions of severe hypoxia. When sections of hypoxic tissue are co-stained with pimonidazole and a hypoxia-inducible protein such as HIF-1α or CA9, distinct and mildly overlapping regions are observed (see Figure 2E), suggesting that the two probes are marking areas of differing severity of hypoxia (253, 545). Although the nature of pimonidazole O_2 sensitivity has been considered reasonably binary (staining is either present or absent), gradients in intensity corresponding to measurable gradients in PO_2 have been reported across the intestinal wall (294). This tissue is reported to be severely hypoxic (see Section VI-G-2), and hence it is possible that differences in staining intensity may be resolved within the reported range (PO_2 0-1.5 kPa). A disadvantage in the use of 2-nitromidazole compounds to identify hypoxic tissue is that staining requires live cells, thus the dye must be injected prior to samples being collected. This limits its use in retrospectively identifying hypoxic areas in tissue specimens already collected or in patient samples. A caveat to the use of immunostaining members of the HIF pathway as a marker of tissue hypoxia is the well-described potential of HIF-1α to be stabilized under oxygenated conditions, most notably during inflammation due to crosstalk with NFκB and direct effects of reactive oxygen species (44, 607). Hence, results obtained using HIF-based immunostaining should always be confirmed with an alternative method.

III. Oxygen delivery *in vivo*

A basic understanding of the delivery of O_2 from the atmosphere to the cytosol is fundamental for interpreting specific tissue O_2 distribution. Moreover, insights into O_2 transport *in vivo* provide a useful perspective for some of the challenges associated with oxygenation of cell monolayers *in vitro*. A point worth clarifying prior to any detailed further discussion is the difference between the terms saturation, concentration and content as commonly used throughout physiology, and partial pressure used universally. In mammals O_2 is transported in two forms: bound to hemoglobin (Hb) and dissolved in blood. Saturation refers to the ***percentage*** of total number of Hb bindings sites saturated with O_2 (4 moles O_2 bind to 1 mole Hb), noting that Hb saturation occurs in continuum until all molecules are saturated. This unit does not strictly measure the concentration of O_2 present (although this can be inferred), but is a good measurement of total blood O_2 content. Although convention is to express components of a chemical reaction as a concentration (in moles per L, for example), O_2 should be reported as **partial pressure** (in mmHg, torr or kPa, Figure 5A) in physiology, noting the final dissolved gaseous concentration in a liquid is proportional to its partial pressure within the corresponding gas phase (Henry's Law (235), taking into account changes in temperature and solubility, Section IV-B). This is because the chemical potential of physiological gases is determined by their partial pressure, and as such they will diffuse down pressure, not concentration, gradients. Content refers to the total amount of O_2, in **ml/100ml**, that

blood can carry, combining that bound to hemoglobin with that dissolved in plasma according to the following equation:

$$cO_2 = ([O_2]_{max} \times [Hb] \times SO_2 \times 0.01) + (H \times PO_2) \quad (1)$$

where the maximal O_2 carrying capacity of blood ($[O_2]_{max}$) is 1.31-1.36 ml O_2/g hemoglobin, otherwise known as Hüfner's constant (110, 194), and *H* is the Henry's Law constant for O_2 (0.023 ml/kPa/100ml blood). Human blood contains on average 14-15 g hemoglobin per 100 ml (194, 604), therefore the O_2 content (cO_2) of fully O_2-saturated (SO_2 ~100%) arterial blood = (1.34 x 15 x 100 x 0.01) + (0.023 x 13) = 20.4 ml/100 ml blood. Note that O_2 content is also expressed in units of concentration (ml/100ml), but differs from the traditional use of O_2 concentration which typically refers to only dissolved O_2 in solution.

With these three terms defined, the disparity between O_2 delivery *in vivo* and *in vitro* becomes readily apparent. In the absence of hemoglobin *in vitro*, O_2 delivery to the cell monolayer is defined largely by the PO_2 of the culture medium directly adjacent and therefore the total available O_2 is considerably lower, e.g. cO_2 = 0.48 ml/100 ml medium under standard atmospheric conditions. The issue of O_2 delivery *in vitro* will be discussed in more detail in Section IV. As cells cannot access O_2 bound to Hb, total O_2 content is not a particularly useful unit to use when comparing *in vivo* and *in vitro* O_2 conditions at the cellular level. Ideally, $[O_2]$ should be defined in units of partial pressure (mmHg or kPa) in compliance with Henry's Law (235). However, the majority of biologists prefer to express gas concentrations *in vitro* as % of atmospheric composition, i.e. *cultured in a standard 37^{o}C, 5% CO_2 incubator*. As O_2 expressed as % ≈ kPa at sea level (see Figure 5A), we have standardized the expression of $[O_2]$ in kPa units in this review.

A. From air to blood

Even before entering the circulation, humidification of incoming air within the trachea reduces PO_2, and the introduction of CO_2 (~5 kPa) within the alveoli reduces this further (Figure 3A). Hence the gradient between atmosphere and alveoli can be described according to the following equations:

$$PO_{2(trachea)} = (P_{atm} - P_{H2O}) \times F_iO_2 \quad (2)$$
$$PO_{2(alveoli)} = PO_{2(trachea)} - (PCO_2 / RQ) \quad (3)$$

where atmospheric pressure (P_{atm}) is 101.325 kPa at sea level, normal water vapor partial pressure (P_{H2O}) is ~6.2 kPa and the fraction of O_2 in inspired air (F_iO_2) is 0.209. Thus $PO_{2(trachea)}$ is normally ~19.9 kPa. As this inspired air mixes with expired air containing CO_2 (PCO_2 ~5 kPa) when respiring with mixed carbohydrate/fat fuel (RQ, respiratory quotient 0.8), the PO_2 is reduced further to ~13.3 kPa. A proportion of venous (deoxygenated) blood from the microvasculature supplying the larger airway and lung adventitia drains into the pulmonary vein rather than the bronchial vein (19, 76). Because of this nuance in lung perfusion, known as pulmonary venous admixture or

shunt, a small gradient exists between the alveoli and arterial PO_2 (usually <1.3 kPa) in healthy adults), spoiling an otherwise remarkably efficient exchange. Taken together, the passage of O_2 from air to the blood results in a gradient of ~9 kPa, the largest such gradient in mammalian physiology.

B. From blood to cytosol

1. Longitudinal gradients within the vasculature

The subject of intravascular O_2 gradients has been reviewed elegantly by Tsai et al. (2003), and as such will only be described briefly in this review. Duling and Berne (1970) were the first to demonstrate experimentally a significant loss of oxygen from the arteriolar circulation. They and many others subsequently established that (i) there is a significant decrease in O_2 between the systemic arterial circulation and arteriolar vessels (43, 123, 124, 263, 325, 459), which is proportional to luminal diameter (124, 500) and inversely proportional to red blood cell velocity/blood flow (295, 584); (ii) the difference between terminal arteriolar and post-capillary venular O_2 is minimal (325, 534), as is the gradient between capillary and tissue O_2 (43, 325); (iii) the next largest tissue compartment outside the lungs in which a significant O_2 gradient is observed is the vascular wall of arterioles (99, 124, 428, 507, 532, 592), which is proportional to the thickness of the arteriolar wall (124); and (iv) A post-capillary diffusional shunt occurs in most microvascular beds, in which a portion of O_2 from saturated arterioles diffuses into venules running parallel (131, 534). Furthermore, measurements of O_2 saturation in red blood cells by absorption at 555 nm (460, 461) confirm that Hb saturation falls proportionally with dissolved O_2 (457). Based on these observations, the arteriolar network appears to plays more of a role in the delivery of O_2 to the tissue than previously considered (500), and there may even be a role for post-capillary venules in homogenizing tissue PO_2 (328). This was first noted following the observation that the relationship between venular diameter and luminal PO_2 was opposite to that described for arteriolar vessels. In other words, blood PO_2 increases between post-capillary and collecting venules (43, 131, 295, 325, 532, 534, 622). However, it must be noted that blood entering the arteriolar network has a PO_2 above the steepest part of the O_2 dissociation curve of hemoglobin (see Figure 4B), and therefore it is hard to reconcile how so much O_2 is transferred to the surrounding tissues from these vessels unless only the transfer of dissolved O_2 is being measured. Moreover, one must also weigh up the influence of the magnitude of the vascular wall PO_2 gradient (the argument in favour of arteriolar O_2 delivery) against the raw number of vessels (capillaries vastly out-number arterioles) when concluding the relative contribution of each type of vessel to tissue O_2 delivery.

2. Radial gradients across and within the vascular wall

The evidence for longitudinal O_2 gradients within the vasculature is complimented by the finding of significant radial, or transmural, gradients across the vascular wall. Radial gradients have been

reported in the rabbit aortic wall (99, 428, 508), cat pial vessels (124), dog carotid arteries (507), dog femoral arteries (410) rat mesenteric arterioles (592) and rat cortical microvessels (622). Elegant work by Niinikoski et al. (428) and Santilli et al. (507, 508) using electrode penetration of the vascular wall demonstrated the existence of a substantial drop in O_2 between the lumen and a very thin tissue layer directly adjacent, attributed by the authors to the endothelium, although the magnitude of this drop has been questioned subsequently (see Section VI-B-1). Nevertheless, a mismatch between intimal and luminal PO_2 would suggest inefficient transfer of O_2 from blood to cell. A physical barrier at the tissue level seems highly unlikely since a high O_2 diffusion resistance of the vascular wall would result in higher downstream PO_2 than observed (592), and O_2 is freely soluble in the plasma membrane (562).

The transfer of O_2 between the lumen and the endothelial cell initially involves the dissociation of O_2 from hemoglobin. Considering the well-characterized O_2 dissociation curve for hemoglobin (Figure 4B), it is clear that arterial blood exists at a sufficiently high PO_2 to prevent substantial O_2 dissociation from hemoglobin, as intended in such conduit vessels. Although this relationship can vary with changes in local pH/CO_2 (the Bohr Shift), it is unlikely that such changes would lead to large dissociations at such a high blood PO_2 (see Figure 4B). Moreover, measurements of luminal PO_2 as referenced above reflect *dissolved PO_2* rather than that stored in hemoglobin (total O_2 content). Thus the large drop in PO_2 between lumen and endothelial cell cannot be explained by limited dissociation from hemoglobin. The relative contribution of vascular wall/tissue O_2 consumption to this large decline is not fully agreed upon, as reviewed elegantly elsewhere (456, 594, 603). There is general disagreement as to whether the vascular wall can consume enough O_2 to account for the large radial gradient detected, with large differences between cultured cells, tissue segments and *in vivo* measurements clouding any consensus (summarised elegantly by Vadapalli and colleagues (603)). Differences in experimental technique and interpretation can certainly account for some of this variation (see Section VI-B-1 for a more detailed discussion), leading to the conclusion that many reported measurements of vascular wall O_2 consumption may be overestimated (456, 603). It therefore seems unlikely that this alone is sufficient to drive the large radial gradients alone. Recent evidence indicates that the major barrier to the transfer of O_2 from the lumen to tissue may in fact be the plasma phase of blood. This was first proposed by Hellums (231) who hypothesised that plasma (in which O_2 is poorly soluble) represents a significant barrier between the red blood cell and endothelium. In this model dissolved O_2 fluctuates as a function of hematocrit, with so-called erythrocyte-associated transients increasing PO_2 near red blood cells and gradients extending in-between. Such theories were corroborated by others (148, 405) and later demonstrated experimentally (22, 184, 328). In large vessels with wall thickness large enough to prevent efficient oxygenation of the vascular cells by diffusion alone, O_2 is supplied via the vasa vasorum as opposed to directly from the lumen (58, 230, 410), as discussed in detail in Section VI-B-1.

3. From vessel to cytosol

The largest O_2 gradient in the vascular network is across the arteriolar wall, implicating these vessels as primary sites for O_2 delivery. However, the smaller overall area of arteriolar networks compared to capillaries dictates that large heterogeneity should therefore exist in tissue PO_2, with large gradients extending between arteriolar vessels. O_2 transport between the capillary and tissue was first addressed by Krogh (316), in his now seminal description of the cylinder model. These relatively simple models tend to oversimplify, since microvascular networks are rarely uniformly distributed (except perhaps in skeletal muscle), but rather display a more 'disorganised' network. Moreover, Krogh's model assumed that longitudinal gradients occur along the length of capillaries, which we now know not necessarily the case. The presence of both longitudinal and radial gradients in the vasculature, and diffusional transfer of O_2 between neighbouring arterioles, capillaries and venules, results in a reasonably narrow Gaussian tissue PO_2 distribution (593). The lack of substantial intra-tissue PO_2 heterogeneity is of physiological significance, since an implication of Krogh's model of tissue O_2 distribution is that tissue close to the venular end of the microvascular network would be at a distinct disadvantage in terms of PO_2. Of course, this viewpoint is still over-simplified as neither tissue perfusion, i.e. the number of microvascular vessels per cm^3 tissue, nor is cellular O_2 demand generally homogeneous, and thus tissue PO_2 gradients can still form especially in larger organs and under uncontrolled tumor growth (as will be discussed in Section VI). PO_2 distribution in different organs/tissues will be discussed in later sections.

IV. Understanding oxygenation *in vitro*

A reoccurring theme throughout this review will be the complex nature of cellular oxygenation *in vitro*. The combined effects of a static layer of medium above an O_2 consuming monolayer of cells, and poor solubility of O_2 leads to the formation of a gradient between the atmosphere and intracellular environment (10, 75, 420). In some cases, this gradient can be substantial enough to significantly reduce intracellular PO_2 below ambient, used herein analogously to head-space to refer to the gas phase immediately adjacent to the medium. Thus intracellular oxygenation, and not ambient PO_2, is the key parameter to consider when designing research under low O_2 environments. This section will address some of the nuances associated with achieving the desired *intracellular* O_2 level *in vitro* to recapitulate levels *in vivo*.

A. What is the PO_2 in room air?

The often quoted 20.9 kPa refers strictly to standard 'dry' atmospheric air (see Figure 5B). However, most cell culture is conducted within a controlled atmosphere of 95% air: 5% CO_2. Moreover, incubators are normally run at 75% humidity (compared to ~50% at room air) to prevent excessive evaporation of medium. Considering this, the actual PO_2 within a standard cell culture

incubator is ~18.5 kPa (at sea level, Figure 5) (377, 420). This value is even lower at high altitude, as the water vapor and CO_2 components are a constant ~47/35 mmHg even under reduced atmospheric pressure. Most atmosphere-regulated cell culture chambers used to perform research under O_2 conditions different from atmospheric alter the PO_2 at the expense of nitrogen, thereby maintaining atmospheric pressure. Moreover, many are engineered to run at slightly higher atmospheric pressure at sea level (101.33-101.78 kPa) to obviate fluctuations in PO_2 due to changes in altitude.

B. Achieving the appropriate dissolved O_2 level *in vitro*

Modulating the gaseous atmosphere with respect to O_2 is straightforward, yet cells do not exist in contact with O_2 in the gas phase, with the possible exception of lung epithelial cells (see Section VI-A). The evolution of red cells as an intricate and highly efficient O_2 transporter bears testament to the poor solubility of this gas in solution. In contrast to CO_2, which reaches equilibrium in solution within minutes, O_2 dissolves too slowly to be overlooked in experimental protocols.

1. Factors affecting O_2 solubility

Efforts to determine O_2 solubility are spear-headed by oceanography and hydrometallurgy communities, with O_2 in solution used for numerous industrial processes. Initially defined by Henry's Law (235) (see Section III-A), the solubility of a gas in pure water is related to the temperature of water and the gaseous pressure. Such relationships have been confirmed more recently, both experimentally and computationally (see Battino (1981) (25) and Ming and Zhenhao (2010) (399) for a review), and are illustrated in Figure 5C. The relationship between temperature, pressure and O_2 solubility is further defined by the Bunsen adsorption coefficient (α, measured in $dm^3.dm^{-3}.kPa^{-1}$). From α, the solubility of O_2 (S_{O2}) is defined here in $\mu mol.dm^{-3}.kPa^{-1}$ by:

$$S_{O2} = \alpha / (\text{molar volume} \times 101.325) \quad [O_2 \text{ molar volume} = 22.393] \qquad (4)$$
$$S_{O2} = \alpha / 2269.9$$

This initial equation was refined further by Benson and Krause (32, 33), in their proposal of *units of standard concentration (c*)*, defined as the concentration of dissolved O_2 per volume of solution. As such, cO_2^* in pure water under standard atmospheric conditions is defined as:

$$cO_2^* = S_{O2} \times (P_{atm} - P_{H2O}) \times FO_2 \qquad (5)$$

Temperature also strongly impacts the PH_2O of a given atmosphere, with higher temperature increasing evaporation and thus increasing PH_2O at the expense of O_2. The salinity of a solution also impacts O_2 solubility, as demonstrated in pioneering work from Sechenov (524) and tabulated for convenience elsewhere (399). With relevance to physiology, the vast majority of experimental work is conducted in solutions of a reasonably similar salinity and thus this factor can be assumed constant. As shown in Figure 5C, the solubility of O_2 in pure water at 37°C is approximately 77% that at 20°C, resulting in ~30% less O_2 in solution. This merits consideration when comparing

experimental data derived at room temperature with that at 37^{o}C. Moreover, with relevance to Section IV-B-2, the equilibration of culture medium from room air to physiological O_2 levels is significantly more time-efficient when the solutions are cooled to 4^{o}C.

2. Equilibration in solution

Mass diffusion was first described by Fick in 1855, in a seminal piece of work from which we now derive Fick's laws of diffusion (153). In the absence of cells, O_2 equilibration between atmosphere and medium follows Fick's second law, and can therefore be estimated using a one-dimension, single compartment diffusion model. This is described by the equation Q=AK((Cs-C)/d) (458), where Q is the rate of O_2 diffusion (µM min^{-1}), A is the surface area (cm^2), K is Krogh's constant, derived from the diffusivity and solubility of O_2, Cs and C are the saturating and desired PO_2 (kPa), respectively, and d is the depth of the medium (cm). To frame this scenario in the context of *in vitro* cell culture, imagine a 90 mm petri dish filled with 20 ml medium and placed within a sealed chamber at a PO_2 of 5 kPa. Using Fick's laws, we can estimate that this medium will take ~2 h to equilibrate to 5 kPa, as shown in Figure 5D, in agreement with experimentally derived values (10, 420). This rate can be increased by dividing the 20 ml into two 90 mm petri dishes, thereby doubling the effective surface area for diffusion. Dissolved O_2 levels achieved in culture medium are a critical and often under-estimated factor in conducting experiments under low O_2 conditions aimed at recapitulating O_2 levels *in vivo*. Thus, addition of poorly equilibrated, overly oxygenated medium to cells pre-adapted to low O_2 conditions may generate a reaction akin to the reoxygenation phenomena observed during stroke and myocardial infarction.

3. Accounting for plastic leaching

The equations described above assume that the only path by which O_2 equilibrates in solution is through the gas-liquid interface. Whilst by far the most important source, plastic is not completely impermeable to O_2, and hence O_2 can diffuse through the sides and bottom of culture dishes to varying degrees. This is largely deemed negligible by most researchers based on the extremely low diffusivity and solubility coefficients of standard polystyrene culture-ware (see Table 1-1). The effect of substrate O_2 permeability on pericellular PO_2, specifically comparing 'impermeable' polystyrene with permeably substrates such as silicone rubber or Teflon membranes, has been demonstrated mathematically (274, 466) and experimentally (659, 690). Notably, Powers et al. (466) incorporated substrate permeability into the most widely adopted mathematical model to determine pericellular PO_2 (672), accounting for the surface area in contact with cells and substrate O_2 diffusivity and solubility. This was intentionally left out in Table 1-2 for greater clarity, but essentially involves an additional α value for the substrate. When monolayer O_2 consumption substantially outweighs O_2 diffusion capacity, using a gas-permeable culture substrate can be an effective way to minimise unintentionally hypoxic conditions. On a separate note, plastic-ware left at room air (PO_2 20 kPa) will accumulate a significant amount of O_2 and this will eventually leach

out when placed in an environment of lower PO_2, thus acting as a O_2 source (558). When plastic equilibrated to 20 kPa was placed within an anoxic water solution, dissolved PO_2 reached ~12 kPa after 60 h (558). Although specific rates of leaching will depend on the plastic composition and its mass/surface, it is highly advised that when working with cells pre-adapted to a specific low PO_2, all plastic-ware (pipette tips, stripettes, petri dishes, culture flasks) be pre-equilibrated at the appropriate PO_2 prior to use. This is of particular importance when working with near anoxic conditions, where even small amounts of O_2 leaching can severely compromise experiments, and we would always recommend measuring PO_2 in the medium/monolayer under such conditions to be confident a sufficiently low PO_2 is achieved and maintained.

C. Accommodating O_2 diffusion and monolayer consumption

One of the most common, and incorrect, assumptions made during *in vitro* research is that intracellular PO_2 and ambient PO_2 are synonymous. The consumption of O_2 by cells, coupled to the poor solubility of O_2 in solution, generates a gradient between the head-space and monolayer proportional to the rate of monolayer O_2 consumption (389). Without compensation, cell cultures may inadvertently experience 'culture-induced' hypoxia by consuming O_2 faster than it can diffuse, as discussed in Section IV-D. Under these conditions, it may become difficult to differentiate the cellular response to physiological and hypoxic O_2 levels, resulting in incorrect interpretations. Compounding this problem has been the lack of technology to accurately monitor intracellular O_2 levels in living cells (see Section II). In lieu of physical measurements, many have relied on an derivation of Fick's second law to predict pericellular PO_2 in monolayers cultured under static conditions (26, 199, 371, 384, 389, 421, 466, 559, 596, 659, 672). By considering oxygen diffusion within a culture dish as a one-dimensional diffusion-reaction, cell boundary O_2 levels can be predicted and, given that O_2 can freely diffuse across the plasma membrane (562), this approximately equates to generalised intracellular O_2 levels (Figure 6A). Although exact equations differ between studies (summarized in Table 1-2), the common critical variables in determining pericellular PO_2 were always, as expected, ambient PO_2, cell density, cellular O_2 consumption and medium height. The most comprehensive equation to date has been put forward by Powers et al. (466), in which the equation by Yarmush and colleagues (672) was adapted to reflect the change in cellular O_2 consumption as a function of ambient PO_2. Common to a number of such studies was the conclusion that hepatocyte monolayers (rabbit (559), human (672) and HepG2/3B cell lines (389)) are most likely severely hypoxic even when cultured under room air, which has subsequently been demonstrated experimentally (see Section VI-E).

These theoretical observations have been corroborated using electrode and fluorescence based probes to experimentally determine pericellular PO_2 in monolayer cultures. Increased cell density has been shown to decrease pericellular PO_2 in monolayers of rat hepatocytes (250, 421), human dermal fibroblasts (581), T-47D breast cancer cells (453), and peripheral blood mononuclear

cells (64). Notably, comparisons between cell types (389) revealed the importance of considering cell-type specific differences in density at confluence in addition to O_2 consumption rate per cell. Although mesangial cells had similar O_2 consumption rates to Hep3B cells (~9 nmol/min/mg protein), vastly different pericellular PO_2 (14.6 vs ~0 kPa at room air) results from differences in monolayer protein content, reflecting morphological differences in cell types (389). The other significant variable, fluid height, has also been confirmed experimentally (77, 371, 389, 421, 581) and is likely the most easily controlled to maintain the appropriate pericellular PO_2. Using the equation first described by Stevens in 1965 (559), one can calculate the required medium height to achieve the desired pericellular PO_2 as recently reviewed (462). Other methods to control pericellular PO_2 include varying the permeability of the base substrate (466, 659) to bypass the need for O_2 to diffuse through the medium, and increasing ambient PO_2 to compensate for monolayer consumption and poor diffusivity (75).

As illustrated in Figure 6B, cellular O_2 consumption rates vary considerably between cell types; from $4x10^{-5}$-0.02 amol O_2 $cell^{-1}$ s^{-1} in red blood cells (179, 510) to as high as 200-500 in cardiomyocytes and hepatocytes (20, 165, 200, 478). These differences between cell types most likely arise from differences in metabolic activity and mitochondrial content/density. As monolayer O_2 consumption is a major determinant of O_2 diffusion and ultimately pericellular PO_2, low consuming cell types are likely to experience much greater intracellular PO_2 than those that consume O_2 more rapidly when placed in the same environment. Sekine and colleagues (521) illustrate this concept by comparing pericellular PO_2 values and oxygen consumption rates in monolayers of hiPSC-cardiomyocytes, rat cardiomyocytes, human aortic smooth muscle cells and cardiac fibroblasts. Cardiomyocyte monolayers consume significantly more O_2 than the non-cardiomyocyte cell types and this is reflected in a much steeper gradient between ambient and pericellular PO_2 (see Figure 6B). Similar results were obtained when comparing medium PO_2 in monolayers of human umbilical vein endothelial cells (HUVEC, low consuming, large size) and human embryonic stem cells (hESC, high consuming, small size) (2). If cellular O_2 consumption significantly outweighs O_2 diffusion capacity, a condition of ‘self-inflicted hypoxia’ may arise at sufficiently low ambient PO_2 (77, 421)(Figure 6B). In reality, since cellular O_2 consumption decreases with ambient PO_2 (88, 211, 441, 443), the accuracy of many of the predictions (which assume a constant rate of O_2 consumption) are limited under extreme hypoxia. It is more likely that under these conditions, intracellular O_2 levels in high-consuming cell types range around a value more conducive to viability, under the control of negative feedback mechanisms orchestrated by HIF (178). In support of this, rat hepatocytes cultured at a PO_2 of 4 kPa stain strongly with the hypoxic marker hypoxyprobe (indicating cytosolic PO_2 below 1 kPa) but retain reasonable viability, suggesting a sufficient supply of O_2 to maintain function (421).

One technique used extensively to alleviate problems associated with poor oxygenation *in vitro* has been the use of 'bioreactor' type culture conditions, in which medium is continuously stirred. Under these conditions cellular oxygenation can occur through convection in addition to diffusion, greatly increasing O_2 supply (172). Medium agitation also forms a critical component of a successful microbial culture system, where bacterial or fungal cells are cultured in suspension at very high densities and therefore require additional oxygenation. Such conditions are not generally appropriate for the majority of adherent cell lines without prior immobilization (see bio-artificial livers for an example (579)), although some success has been obtained using roller bottle technology, in which adherent cells are cultured on the side of a cylindrical device placed horizontally on an orbital shaker (175, 462). Furthermore, microfluidic perfusion has received increasing attention in recent years and also solves several problems associated with monolayer oxygenation, especially when constructed using plastic with a high O_2 permeability coefficient. Many of these techniques can create adequate monolayer oxygenation even though more effort and expense is required,, and thereby provide more accessible means of altering existing culture conditions to improve oxygenation.

D. Evidence for compromised oxygen delivery *in vitro*

As eluded to above, the concept of 'self-inflicted' hypoxia is not restricted to theory. Confluent HepG2 cultures rapidly (within 1 h) deplete medium PO_2 to <0.1 kPa even at an ambient PO_2 of ~20 kPa. In these studies, an hypoxic status was confirmed by continuous secretion of the HIF-target protein erythropoietin and an increase in lactate production, indicative of a switch from oxidative phosphorylation to glycolysis (659). Similar observations were made by Metzen et al. (389) and Bhatia et al. (421) in HepG2 and rat hepatocytes, respectively. Compromised O_2 delivery *in vitro* is not confined to hepatocyte cultures, since large culture O_2 gradients have been reported using a number of different cell types under standard culture conditions and low ambient PO_2 (see Table 1-3). Traditional micro-manipulator mounted electrode methodologies have largely been superseded by fluorescence and phosphorescence-based techniques, which can provide a more accurate determination of pericellular PO_2 in culture dishes. Using immobilized luminescent dyes (see Section II-C), large culture gradients have been demonstrated in cultures of human embryonic stem cells (hESC) (2, 320) and Caco-2 intestinal epithelial cells (690). Meanwhile, cell-permeable phosphorescent nanoparticles have been used to monitor intracellular (cytosolic) PO_2 in mouse embryonic fibroblasts (MEFs) (151), cortical neurons (114), and in HUVEC (75). Hence intracellular or pericellular PO_2 provides a more accurate parameter when translating *in vitro* experiments to an *in vivo* setting.

E. Maintaining the appropriate environment for the duration of culture

Culture of mammalian cells under reduced O_2 environments has generally been undertaken using three types of equipment; (i) an airtight, gassed chamber placed within a standard CO_2 incubator, (ii) a tri-gas incubator, or (iii) a dedicated O_2-regulated workstation, with cost and infrastructure requirements increasing accordingly. The former is most widely used due to the relatively low price and ease with which it can be accommodated into existing culture infrastructure, although dedicated chambers generally have limited multi-user space and accessibility. Tri-gas incubators are variations of standard CO_2-regulated cell culture incubators in which an additional N_2 gas input is provided, with which the PO_2 can be manipulated to a required level. Tri-gas incubators offer increased capacity for multi-users, especially units equipped with independent, modular compartments. However, both these types of equipment suffer from an important disadvantage when used for long-term culture of cells under defined low O_2 conditions. Cells need to be removed from the chamber or tri-gas incubator in order to feed, monitor and perform any required sub-culture or experimental treatments. Thus, cells are re-exposed to hyperoxic atmospheric levels of O_2. Such fluctuations in the resultant pericellular PO_2 induce a phenotype distinct from that of cells maintained long-term under a defined level of O_2 (289, 437, 640). Indeed, direct comparisons between culture in tri-gas incubators and dedicated O_2-regulated workstations demonstrate that repeated re-exposure to atmospheric O_2 levels during culture is detrimental to human mesenchymal stem cell growth (289). Medium and pericellular PO_2 require hours to re-equilibrate to a required PO_2 level even when only transiently exposed (~5min) to atmospheric levels (320, 404, 690). Such technical problems associated with in vitro cell culture mimic ‘reperfusion injury’ common following restoration of cerebral or myocardial blood after infarction. Re-exposure to atmospheric PO_2 can be avoided completely by using a dedicated O_2-regulated workstation, where access to cells for sub-culture and treatment is permitted through gas-tight glove ports and transfer chambers. It is for this reason that we strongly recommend that any work investigating the effects of prolonged culture under well-defined low PO_2 be conducted using a dedicated O_2-regulated workstation.

V. Cellular oxygen sensing - when is low O_2 hypoxic?

Having established the difficulties in accurately predicting and controlling monolayer oxygenation *in vitro*, a consideration for how cells sense the difference between normoxic and hypoxic PO_2 is now relevant. The question posed relates to the understanding that physiological oxygenation varies by so much *in vivo* that the use of terminology like hyperoxia, normoxia and hypoxia should be used contextually rather than absolutely, and should always be defined quantitatively for better interpretation. Each cell type has unique responses to a reduction in available O_2. For example, endothelial cells release vascular endothelium-derived growth factor (VEGF) and nitric oxide (NO) to initiate vasodilation and angiogenesis in an attempt to increase local blood flow, thereby normalising tissue O_2 distribution (351, 536, 630). A more generalised ‘hypoxic’ response is

orchestrated predominantly by hypoxic-inducible factors (HIFs), with additional contributions from less well defined pathways. The relevance of these mechanisms in the context of *in vitro* normoxia remains to be defined fully, since they have evolved to respond to acute reductions in PO_2 within (patho)physiological levels, and not the substantial reductions imposed in the majority of studies *in vitro* (room air to 1-3 kPa). Thus the study of cellular phenotypes under low O_2 generally requires culturing cells for a sufficient period to allow *adaptation* to the new baseline [O_2]. Several excellent articles have reviewed the general and state-of-the-art literature concerning how cells sense O_2 (476, 516, 522, 575), and as such only the most well established cellular O_2 sensors will be covered here. Particular emphasis will be placed on their relevance under physiological O_2 levels in terms of the PO_2 at which they have been shown to be active. This will be followed by a brief discussion of how the duration of exposure to low O_2 can influence the interpretation of experimental data.

A. Cellular oxygen sensors

1. Hypoxia-inducible factors

Several excellent reviews have been published on the physiology and biochemistry of HIFs (472, 504, 516, 522), and therefore HIFs will only be briefly discussed. HIFs are evolutionarily conserved transcription factors expressed in all eukaryotic organisms as 3 α isoforms (HIF1-3) and a β subunit. The vast majority of research to date has focussed on HIF-1, which regulates the acute and reactive phase of the hypoxic response in most cell types (257, 549). Whilst the β subunit is constitutively expressed and not actively degraded, the α subunit is post-translationally modified through O_2-dependent hydroxylation at prolyl resides (by prolyl hydroxylases, PHD), which targets the subunit for degradation (35, 134), or at asparagine residues by factor inhibiting HIF-1 (FIH) which interferes with its binding to transcriptional co-activators (238, 322, 323). Thus, when intracellular O_2 availability decreases these enzymes are no longer able to hydroxylate HIFα subunits resulting in the formation of a transcriptionally competent stable α/β dimer. This can then translocate to the nucleus where it binds hypoxia-response elements initiating the transcription of hypoxia-sensitive genes. Such genes encode proteins such as vascular endothelium-derived growth factor (VEGF) (162, 536), erythropoietin (EPO) (630) and glucose transporter 1 (GLUT1) (128), which function collectively to either increase O_2 delivery or reduce cellular O_2 consumption. It was traditionally considered that a large degree of redundancy exists between HIF-1α and -2α (549, 667), which itself has fewer selective targets (120, 193, 257, 444, 569, 599, 634). However, recent pan-genomic screens (503, 514, 515) have identified a large number of HRE binding sites with distinct isoform binding affinities, despite an identical core recognition sequence (RCGTG). Such variance may represent different topographical chromatin binding locations, affinity for HIF-β binding partner and/or the involvement of other transcription factors, most notably AP-1 (321, 503). Comparatively little is known about HIF-3α, but it is thought to act as a negative regulator of HIF-1 and -2 (219).

699

700 The K_m for O_2 of the reaction catalysed by PHD ranges between 140-250μM (~10-20
701 kPa) (243, 517), and thus O_2 may be a rate-limiting substrate under atmospheric O_2 conditions.
702 Despite this, HIFs are not constitutively stabilized in cells at physiologically normoxic levels (on
703 average 5 kPa), otherwise organisms would have no reactive response to further reductions during
704 genuine hypoxia. Persistent stabilization is prevented by HIF-dependent negative feedback
705 mechanisms, including specific interactions with microRNA 210 (631) and reactive oxygen
706 species (254), upregulation of prolyl hydroxylases 2 and 3 (36, 106, 178, 372) and the global
707 impact of HIF-induced proteins to increase intracellular O_2 levels. Thus, any reduction in O_2
708 availability is addressed by transient stabilization of HIF-1α, and the upregulation of associated
709 regulated proteins, followed by pathway normalization at a new baseline reflecting the altered O_2
710 levels. A question that remains unsolved in HIF hydroxylation is the concept of 'range finding'; that
711 oxygen-dependent regulation of HIF is observed in a variety of tissues existing across a huge
712 variety of O_2 levels (476). The high K_m of PHD enzymes for O_2 has led many to conclude that
713 absolute O_2 levels are perhaps of secondary importance in the mechanism by which cells sense
714 O_2, and that complimentary signals may further modulate hydroxylase activity. To date, alternative
715 signals include reactive oxygen species (74, 378), intracellular [Fe^{2+}] (306), and competing
716 hydroxylase substrates (91). Inclusion of these additional parameters in the mechanism of HIF O_2
717 sensing creates an increasingly complex model which may offer insight into the 'range finding'
718 capabilities of the system (476), a concept critical for defining physiological normoxia.
719

720 *2. Mitochondria and/or superoxide*

721 Given their central role in O_2 homeostasis and intracellular signaling, mitochondria are prime
722 candidates for intracellular O_2 sensors. Mechanisms by which these organelles are thought to
723 transduce changes in O_2 levels revolve around changes in either intracellular redox or energy
724 states (or both) (74, 633). Limitations in O_2 supply would logically lead to reductions in
725 mitochondrial ATP generation which subsequently activates AMP-dependent protein kinase
726 (AMPK), a cellular energy sensor (220, 556), although some have argued this occurs due to
727 reactive oxygen species generation and not changes in ADP/ATP (132). AMPK in turn targets
728 several transcription factors to repress fatty acid biosynthesis and upregulate proteins involved in
729 glycolysis, resulting in a metabolic shift away from O_2 consuming oxidative phosphorylation (221)
730 Pharmacological inhibition of the electron transport chain (ETC) can mimic hypoxic
731 responses (121, 392), although others report an abolition in O_2 sensing following ETC
732 inhibition (635). One major limitation to the energy state hypothesis of mitochondrial O_2 sensing is
733 that the P50 of cytochrome C oxidase for O_2 is ~0.07 kPa (181), and thus ATP production would
734 not be expected to decrease until cytosolic PO_2 fall very low. However, some have suggested that
735 the affinity of cytochrome C oxidase for O_2 is modified substantially by cellular reducing agents
736 (GSH and NAD(P)H), suggesting that O_2 may become rate-limiting at much higher levels than

previously predicted (633, 653). The generation of superoxide anions by the ETC has formed the basis of intense debate in the context of mitochondrial O_2 sensing, with both decreases and increases in mitochondrial superoxide ($O_2^{\cdot-}$) generation reported to mediate O_2 sensing. On one side, reductions in O_2 availability would logically result in reductions in $O_2^{\cdot-}$ production, which in turn would cause the cytosolic environment to become more reduced, affecting many redox-sensitive pathways (15, 480, 641). Conversely, others have reported a paradoxical increase in superoxide generation in mitochondria during hypoxia (74, 206, 207, 373, 450, 609, 636), resulting from enhanced electron leak within the ETC.

Mitochondria are not the only source of reactive oxygen species within the cytosol, and indeed other enzymatic sources have been implicated as O_2 sensors. Since NAD(P)H oxidases (NOX) function to generate reactive oxygen species with a reported P50 for O_2 of ~1.7 kPa (31), it is tempting to speculate that NOX serve as a key cytosolic O_2 sensors (633). Indeed, mice lacking an essential subunit of the NOX2 isoform ($gp91^{phox}$) exhibit impaired O_2 sensing (168), although this remains controversial (14, 633). Both increases (229, 375) and decreases (100) in NOX-derived reactive oxygen species generation have been reported during hypoxia.

3. Gaseous messengers

A new O_2 sensing mechanism has emerged in recent years focusing on the interplay between two physiological gaseous messenger molecules, carbon monoxide (CO) and hydrogen sulphide (H_2S) (468). The effects of CO on hypoxia-induced ventilation have been well known for 50 years (352), yet the finding that it is produced endogenously highlighted its possible involvement in O_2 sensing. CO is produced by heme oxygenases, of which there are two principle isoforms: inducible (HO-1) and constitutively expressed (HO-2) (369, 542). Importantly, production of CO is O_2 dependent (652) with a P50 of 8.7 kPa (683), placing this potential sensor within the physiological range. CO was found to inhibit the activity of cystathionine-γ-lyase (CSE), the enzyme responsible for H_2S synthesis, and hence H_2S production is increased when HO-2 becomes substrate limited during hypoxia (683). In archetypical O_2 sensing cells, e.g. type I cells of the carotid body, it is proposed that H_2S initiates depolarisation through inhibition of Ca^{2+}-activated K^+ channels (340) and increasing Ca^{2+} influx (56). Less is known about the role this CO-H_2S sensing system functions in non-excitable, non-specialised cell types.

B. Exposure or adaptation – Does timing matter?

With the exception of the HIFs, the above O_2 sensing mechanisms have been described in the context of whole body physiological reactions to hypoxia, and therefore have an implied negative feedback loop whereby a plethora of actions instigated by the sensing of hypoxia act in conjunction to increase tissue PO_2, to alleviate input stimuli. There is little information on how these mechanisms function in chronically hypoxic environments. The general consensus is that HIF-1α

actions peak after 12-16 h exposure to low O_2, and then normalize by 24 h to a new set-point (62, 178, 249, 630). In contrast, any stabilization of HIF-2α has been shown to require considerably longer (>72 h) (249, 355). Since these transient responses to reduced O_2 availability are unavoidable during transfer from room air to physiological normoxia, it is critical that experiments aimed at recapitulating physiological conditions enable cells to adapt long-term. Importantly, this should include the time required for the expression of any induced protein to also normalize. A global analysis of protein half-life reveals a mean half-life of 46 h (520), and thus the adaptation time required to circumvent these initial responses to reduced O_2 can be estimated. We have observed that at least 5 days are required for a phenotype to stabilize in human endothelial cells when transferred from room air to a PO_2 of 5 kPa during culture (75, 290), with culture beyond this period inducing no further alterations in cell phenotype (75). To avoid misinterpretation of experimental data, we suggest that cells are cultured for as long as possible (at least 5 days) under physiological normoxic conditions.

VI. Replicating normoxia *in vitro*

Significant efforts have been undertaken to better recapitulate a physiological milieu *in vitro*, with particular focus on the physical environment in which cells are cultured. For example, numerous studies have characterized the phenotype of endothelial cells exposed to laminar flow (80), or vascular smooth muscle cells (481) and renal mesangial cells (198) exposed to cyclic biaxial stretch, or dermal fibroblasts in 3D extracellular matrix culture (197). However, few studies have carefully considered the gaseous composition of the culture environment. The following sections provide an overview of current understanding of PO_2 in specific tissues *in vivo* and summarize the key studies aimed at replicating these conditions *in vitro*. We focus specifically on investigations in mammalian cells under quasi-physiological PO_2 (not hypoxia) and long-term culture conditions (2-12 kPa, >24 h) to avoid confusing physiological phenotypes with those associated with relatively rapid adaptive responses to reduced O_2 availability. However, it is worth noting that when reviewing this literature, in most cases only ambient PO_2 is considered and therefore many observations may not describe the ideal physiologically normoxic phenotype. Furthermore, tissue PO_2 distribution is considerably heterogeneous in both space (regional differences in perfusion or consumption) and time (changes in metabolic activity). The data quoted herein therefore often represent one observation across a range of potential values, and should be interpreted accordingly. All measurements of tissue PO_2 discussed in this review have, to the best of our knowledge, been performed on intact preparations kept *in situ* to maintain the endogenous blood supply and minimise interference from the atmosphere.

A. Lungs

In the airways, PO_2 in the gaseous phase can be predicted accurately, and thus concentrations within the respiratory tract are known with high certainty (see Section III-A and Figure 3).

Consistent with the large area occupied by the bronchi, bronchioles and alveoli, whole organ PO_2 maps (see Figure 7B) conducted with MRI scanning using ^{3}He polarisation (3) reveal average PO_2 values of 14.5 (107), 13.4 (649), 13.6 (396), 14 (216), ~12.7 (217), and 13.5 kPa (407) in human subjects, largely reflecting inspired air PO_2. Whole lung PO_2 is lower in chronic smokers (216), but does not differ in patients with heart failure, although the alveolar-arterial gradient (see Section III-A) is significantly higher in these patients suggesting a less efficient gaseous transfer (407). These values closely reflect values obtained in experimental animal models, where mean PO_2 values of 11 (156), 14.7 (682) and 19.6 kPa (107) have been reported in porcine lung, 17.1 kPa in rat lung (85) and 14.7 kPa in rabbit lung (681). Regional heterogeneity in PO_2 measurements throughout the lung was common in all the above studies, consistent with the known variability in lung perfusion and gas exchange. This, coupled with a small image acquisition field of view, can lead to lung PO_2 values skewed by sampling in highly or poorly perfused regions. Encouragingly, lung or alveolar PO_2 values determined by ^{3}He-MRI correlate very well with traditional methods used to determine alveolar PO_2, in which PO_2 in the expired gas is related to arterial PO_2 and PCO_2 using equations 2/3 (see Section III-A) (217, 407).

The need to transition into the aqueous phase within the airway surface liquid (ASL) for bioavailability make estimates of how a gaseous PO_2 of 13-18 kPa translates into cytosolic PO_2 within airway epithelial cells ambiguous. The average ASL depth in healthy human bronchi is 15-80μm (273) and is therefore unlikely to present a significant barrier to O_2 diffusion. To our knowledge, no measurements of ASL PO_2 in healthy bronchi have been reported, and thus we are left to interpolate lung epithelial PO_2 based on ASL depth. Worlitzsch et al. (664) elegantly demonstrated that increased ASL depth (up to 800μm) in patients with cystic fibrosis resulted in a mean mucosal PO_2 of 2.5mmHg (0.3 kPa). Such hypoxic conditions were associated not only with increased ASL, but also with increased epithelial cell O_2 consumption, and facilitated the early pathogenesis of anaerobic *P. aeruginosa* infections (664).

Beyond the epithelia, the lung connective tissue largely consists of fibroblasts whose role it is to maintain the structural integrity of the organ. Intriguingly, adventitial PO_2 has been reported significantly lower than the average lung PO_2 (13-14 kPa) as measured by ^{3}He-inhalation. Using microelectrode penetration, 'peribronchial' PO_2 values of 5.7 kPa were recorded in patients inspiring >50% FiO_2 (326). Similar values have been described in porcine (5.1 kPa (236), 5.8 kPa (205)), canine (~6.5 kPa (282)) and rat (5.1 kPa (487)) experimental models. In the latter study (487), an implantable EPR resonator was used in conjunction with O_2-sensitive EPR probes to monitor deep tissue PO_2 in real time, confirming measurements using microelectrodes. Anatomically, the site most often sampled by microelectrode penetration lies ~1cm distal to the tracheal bifurcation, near to the peribronchial lymph node. Kamler and colleagues (282) demonstrated that ligation of the aortic stem of the bronchial artery dramatically reduces

peribronchial PO_2 in this region, even when breathing was maintained (Figure 7C). This, coupled with the observation that peribronchial PO_2 does not increase proportionally with FiO_2 (unlike arterial PO_2) (236), suggests that lung adventitial PO_2 is critically dependent on blood perfusion from the bronchial artery, and does not receive O_2 supply directly from the airways. How representative this highly sampled region is of non-airway lung tissue as a whole remains to be established, although it has been used as a measure of overall tissue oxygenation following lung transplant and correlates well with improved outcomes (282, 425, 431).

Unlike most other cell types, lung epithelial cells experience a high PO_2 physiologically and are only separated from gaseous O_2 by a thin layer of ASL. These conditions have been recapitulated *in vitro* in the form of air-liquid interface culture, as illustrated in Figure 7D. First introduced in the late 1980's by Wu, Kim and colleagues (6, 7, 645), this technique has been shown to be essential in maintaining primary cultures of tracheal and bronchial epithelial cells in a differentiated state *in vitro*. Principally, the pseudostratified phenotype observed *in vivo* can be recapitulated *in vitro* after ~7 days culture at the air-liquid interface (5, 645), with cells typically differentiating into ciliated and granulated mucus-secreting epithelial cells. Other responses to culture at the air-liquid interface include: increased Na^+ transport via short-circuit current (I_{sc}) (278), restoration of epithelial barrier function (190) and a transcriptional profile more comparative with fresh tracheal/bronchial epithelial cells *in vivo* (126, 454), even in response to cigarette smoke inhalation (381). More recently, culture at the air-liquid interface has been used as a tool to differentiate inducible pluripotent stem cells into mature airway epithelial cells (470, 662). This method of culture clearly has profound impact on the phenotype of airway epithelial cells, very accurately recapitulating that observed *in vivo*. However, we could not identify a publication employing air-liquid interface culture in which the PO_2 was implicitly defined. When the immortalised epithelial A549 cell line was cultured long-term at a PO_2 of 13 kPa, an increased sensitivity to copper oxide nanoparticles was observed paralleled by a decrease in cellular antioxidant defences (317). Although pericellular PO_2 may be lower than 13 kPa in this model, thus poorly representing physiological normoxia, this study provides important insight into the potential of employing culture under physiological normoxia to evaluate environmental toxicity in models of airway damage. Standard incubator conditions (~18.5 kPa , 5 kPa CO_2) reasonably reflect upper tracheal PO_2, but bronchiolar PO_2 can drop as low as 13 kPa. Thus careful control of ambient PO_2 during air-liquid interface culture may yield interesting insights into phenotypic differences related to PO_2 between epithelial cells along the length of the respiratory tract.

B. The vasculature

The mechanics of O_2 distribution in the vasculature have already been covered in Section III-B, and this section aims to further discuss the absolute PO_2 values measured in the vascular network. In estimating PO_2 values experienced by different cell types in the vascular wall (endothelial,

smooth muscle, fibroblasts), it is critical to assess the level of O_2 within the wall tissue itself. An excellent review by Tsai, Johnson and Intaglietta (594) summarizes the distribution and gradients of O_2 in the microvasculature, and we have here integrated their summaries and conclusions with recent research findings. Current understanding of the PO_2 levels in vessels related to specific tissues will be discussed in the context of the relevant tissue.

1. The macrovascular wall

Section III-B-2 introduced the concept of radial gradients across the vascular wall, which are particularly prominent in larger arteries such as the aorta and carotid arteries. O_2 supply to the endothelial layer occurs largely by diffusion from the lumen (containing oxygenated blood PO_2 ~12 kPa), noting that the thickness of the walls of such vessels can severely limit adequate delivery. As such, vessel wall PO_2 decreases significantly between the luminal and medial layers, as first reported nearly 50 years ago (410, 428). By advancing a tissue-penetrating Clark-type electrode perpendicular to the vessel wall, as illustrated in Figure 8B, a pattern of wall oxygenation in large arterial vessels such as the aorta, femoral and carotid arteries is clearly evident. Penetrating the intimal layer was associated with a prominent, rapid reduction in PO_2 in the rabbit infrarenal aorta (237, 330, 428, 509) and abdominal aorta (505, 506, 508), pigeon abdominal aorta (214), and dog carotid artery (507). In such studies, PO_2 in the intimal layer reached an average of 3-5 kPa, representing a drop of 5-7 kPa from the lumen. Others reported no such sudden drop in similarly sized vessels and preparations (23, 57, 97, 99, 279, 691), instead reporting gradual declines in PO_2 across the inner third of the wall, reaching a similar nadir within the medial layer. Such discrepancies have been attributed largely to differences in methodology, including (i) the nature of vessel preparation and maintenance, with contrasting PO_2 profiles observed when the vessel is left *in situ* (237) versus superfused with physiological solution at a controlled PO_2 (97) and (ii) the method of electrode advancing, with many using 'vibration mounting' apparatus to minimize tissue distortion. Some (509) have postulated that the resonance created by such devices can interfere with the laminar flow patterns close to the vessel wall, considered critical in forming the aforementioned O_2 diffusion barrier. In contrast, others (98) have suggested that the sharp decline in PO_2 at the intimal layer is an artefact resulting from the use of large-diameter electrodes. Whether sharp or gradual, experimental evidence is clear that vessel wall PO_2 declines proportional to distance from the lumen (in vessels devoid of vasa vasorum, see below).

Aside from this discrepancy, remarkable conformity exists in the reported minimal vessel wall PO_2. The nadir within the medial layer shown in Figure 8B-C occurs at 50-70% of the wall thickness away from the adventitia (~200μm in a rabbit thoracic aorta) and reaches a PO_2 of 3-4 kPa (23, 237, 279, 330, 428, 505, 506, 507, 508, 509). This value has been shown to correlate negatively with total wall thickness (97, 279), and hence conditions in which vessel wall hyperplasia occurs can result in significant reductions in medial PO_2, as observed in alloxan-

induced diabetes (505), atherosclerosis (279), hypertension (99, 506), after insertion of vascular grafts (330, 509) or stents (508) and after balloon de-endothelialization (691). Reduced medial PO_2 in such conditions has been attributed to an altered bioenergetic profile in resident (and migrating) cells (330) as well as biophysical considerations such as increased wall stress (508) and compression of the vasa vasorum (57, 508). Medial hypoxia has been demonstrated *in vivo* in rabbits with late-stage atherosclerotic plaques in the aortic arch and thoracic aorta using the biochemical hypoxia marker 7-(4'-(2-nitroimidazol-1-yl)-butyl)-theophylline (NITP) (41). In this study, plaques >400µm diameter exhibited hypoxic regions ~200µm in diameter and 200-300µm from the endothelial/intimal surface, with an estimated PO_2 of 0.5 kPa. Using similar probes ([^{18}F] EF5 and [^{64}Cu]ATSM), hypoxic regions have been visualized within the media of athero-susceptible transgenic mice (427, 537) and rabbits (426). Atherosclerotic plaques occur preferentially in regions of the vasculature that experience oscillatory blood flow, such as the inner curvature of the aortic arch and carotid bifurcation (370) and lower medial PO_2 has been measured in such regions using microelectrodes (507). It is therefore possible that medial hypoxia precedes and facilitates atherogenesis in such regions, rather than occurring subsequent to plaque development and wall thickening.

Limited data are available on PO_2 profiles in large arteries in humans, as measurements are invasive and difficult to perform in humans. However, Vorp and colleagues (620, 621) performed microelectrode measurements of PO_2 within the abdominal aortic wall of patients with aneurysms. Although reported measurements were normalized to luminal PO_2, interpolation based on an average aortic luminal PO_2 of ~11 kPa suggests that the aortic aneurysm wall PO_2 was ~7 kPa in the absence of intraluminal thrombi, falling to ~2.5 kPa when a thick thrombus was evident (620). Although the exact topographical location of the PO_2 sampling point was not reported, an aortic wall PO_2 of ~7 kPa is similar to values measured in animal models (279, 505, 506, 508).

The walls of large blood vessels can be thick enough in diameter to merit their own blood supply, and in arteries these vessels are termed the vasa vasorum. The extent and nature of such vessels is critically dependent on the wall thickness. Peri-adventitial vasa can exist in moderately sized arteries such as the femoral or carotid, whereas vasa penetrating into the media are thought only to occur beyond a critical threshold diameter (>500µm or 29 lamellar units, irrespective of species or vessel characteristics) (661). In the context of vessel wall PO_2 distribution, the presence of vasa vasorum is critical in defining adventitial PO_2, as outlined in Figure 8C. An upward inflection beyond the 'medial nadir' in the PO_2 profile of a vessel wall is characteristic of a second source of O_2 diffusion from the outer layers. Arteries confirmed histologically to be devoid of adventitial vasa demonstrate a continuous decline in PO_2 between the lumen and adventitia (97, 99, 279), and experimental disruption of vasa vasorum patency (surgical or resulting from increased wall stress secondary to increased blood pressure) in those vessels possessing peri-adventitial vasa results in

a significant reduction in adventitial PO_2 (23, 57, 330, 508, 509, 691). Moreover, increases in aortic tissue PO_2 in response to vasodilatory compounds (adenosine and epinephrine) applied to the adventitia have been attributed to vasa vasorum vasodilation (58). Notably, adventitial and medial neovascularization often occurs in response to vessel injury (413), and Lee et al. (330) showed that decreased adventitial PO_2 following insertion of an anastomotic graft can resolve over time in conjunction with the histological appearance of new medial vasa.

2. The microvasculature

The vascular network between an artery and vein, encompassing arterioles, capillaries and venules, is the prime region for O_2 exchange, as evidenced by the significant difference in blood PO_2 between an artery and vein (~6-7 kPa). This longitudinal gradient was first highlighted in Section III-B-1, and is the focus of previous reviews (456, 594). As such, only an overview of blood PO_2 within the microvascular network will be included here. Microvascular networks are present in all organs, and we provide an overview of blood PO_2 in an 'idealised' microvascular network comprised of (i) a feeding artery, (ii) 1^{st}, 2^{nd} and 3^{rd} order arterioles, (iii) a capillary bed, (iv) 1^{st}, 2^{nd} and 3^{rd} order venules and (v) a collecting vein (Figure 9A). The majority of studies of microvascular PO_2 distribution have focused on 3 experimental preparations: the hamster skinfold/cheek window, rat cremaster/spinotrapezius muscle and rat pial/cortical microvasculature. Their minimally invasive nature and the ability to monitor intravascular PO_2 in live animals are major advantages of such models. A summary of studies for each experimental model is provided in Figure 9B. Blood enters the microvascular network at an average PO_2 of ~9 kPa and rapidly declines along the arterioles to reach a capillary PO_2 ~5 kPa. As described by Tsai et al. (594), such a finding suggests that arterioles, and not capillaries, are the major source of O_2 delivery in most microvascular beds. This may be true for hamster cheek pouch and rat muscle preparations, but reports in rat pial microvessels indicate that intravascular PO_2 in pre-capillary arterioles is still ~7-9 kPa, and only falls substantially once it has entered the capillary networks (622). However, recent evidence using 2-photon time resolved phosphorescence (500) has refuted such claims, demonstrating that arterioles in the mouse cerebral cortex are responsible for ~50% of O_2 delivered to the underlying parenchyma. These authors postulated that the expansive capillary network may act as a reserve mechanism for O_2 delivery during times of increased activity (500).

Unlike large vessels, the presence of radial gradients and their contribution to tissue PO_2 in arterioles is less well defined. Many studies report differences between intravascular and peri-arteriolar PO_2 (260, 531, 585, 591) which appear proportional to the blood PO_2 and luminal diameter (594). Such differences are in the order of 2.5-3.5 kPa in large (A1) arterioles (531, 591) and ~1.5 kPa in moderately sized (~40μm diameter) arterioles (260, 531, 585). In contrast, others have observed an arteriolar wall O_2 gradient of only a few mmHg (1.4 mmHg/0.2 kPa per μm (122, 123), 1.2 mmHg/0.16 kPa per μm (527, 623)). Pittman (456) proposed a possible

explanation for this discrepancy, citing misinterpretation of phosphorescence quenching data due to O_2 consumption by the probe itself. Attempts to remedy these differences have subsequently been made by adapting the methodology (182, 183, 185). Unusually high vascular wall consumption, the reasoning put forward to explain large radial arteriolar gradients (594), was also refuted by Vadapalli et al. (603). With relevance to estimating physiological normoxia for cells resident within the microvascular wall, we believe that intravascular (blood) PO_2 most appropriately represents this value.

3. The human umbilical vein

One of the most well studied and widely utilized vascular cell type *in vitro* are human umbilical vein endothelial cells (HUVEC). However, its unique physiology (fetal, venous tissue carrying oxygenated blood at a low flow rate) means it does not reflect normal artery/vein, macro/microvascular characteristics. Unlike many other tissues in the human body, the umbilical cord can be accessed relatively easily/non-invasively for blood collection and gas analysis, albeit *ex vivo*. It is therefore fortunate that endothelial cells isolated from these vessels have been used extensively in vascular biology *in vitro*, allowing for an accurate and well characterised transition to physiologically normoxic cell culture. A meta-analysis of data extracted from a number of studies reporting umbilical blood PO_2 (102, 109, 298, 313, 347, 376, 465, 486, 576, 605, 675) reveals a mean blood PO_2 of 3.8 and 2.4 kPa within umbilical veins and arteries, respectively. Although collected *ex vivo* under room air, this has been shown not to increase measured O_2 levels in umbilical blood (382). Interestingly, umbilical vein PO_2 decreases throughout gestation from ~6 kPa at 20 weeks to ~4 kPa at 36 weeks (639), and final PO_2 at birth is significantly affected by the method of delivery (spontaneous vaginal vs caesarean) (109, 313) and position of the fetus (breech) (102). The human umbilical vein is a macrovessel of 2-3 mm diameter with an average wall thickness of 430µm and a moderate adventitial layer (344). Although not studied, based on these dimensions, it seems likely that a significant radial gradient would be apparent across the length of the wall (Section III-B-2), and thus physiological normoxia in smooth muscle cells in these vessels may be lower than that measured in the endothelium (~3.5 kPa (75)).

4. Endothelial cells

The physiological response to hypoxic tissue O_2 levels is an initial vasodilation followed by the induction of angiogenesis, with both responses increasing O_2 delivery and thereby alleviating the harmful effects of hypoxia (536, 571). Given the primary role of endothelial cells in orchestrating such responses, it is no surprise this cell type has received considerable attention in the context of low O_2 physiology (393, 472, 523) as summarized in Table 1-4. We recently described a well-defined model to recapitulate physiological normoxia in cultured human venous and arterial endothelial cells (75, 290, 291). Using a phosphorescent nanoparticle probe to monitor intracellular PO_2 in living cells (Figure 10A), we established that culture at a PO_2 of 5 kPa was required to

compensate for monolayer O_2 consumption and recapitulate a physiologically normoxic intracellular environment of 3.5-4 kPa (measured in umbilical venous blood, see above). In order to maintain such conditions in different culture apparatus (6-, 24- or 96-well plates) with varying surface area, volume was adjusted to achieve similar surface area: medium depth ratios. For example, 100µL in a 96-well plate has roughly the same surface area: medium depth as 500µL in a 24-well plate.

The importance of distinguishing between ambient and intracellular PO_2 was further highlighted by the different absolute P_{50} (akin to E/IC_{50}) (633) values for HIF-1α stabilization when plotted against each O_2 parameter (Figure 10C) (290). When ambient PO_2 is used, the resulting P_{50} value (4.3 kPa) is not consistent with the known PO_2 distribution in the majority of the microvasculature nor in tissues, where a PO_2 4-5 kPa would be considered normoxic and hence minimal HIF-1α stabilization should be apparent. In contrast, use of intracellular O_2 levels results in a P_{50} value of 2.3 kPa, a level more consistent with what one may expect to observe in the hypoxic vasculature *in vivo*. Under these defined conditions, we have shown that the induction of nuclear factor E2-regulated factor 2 (Nrf2) regulated antioxidant gene expression in response to oxidative stress was markedly attenuated in HUVEC (Figure 10D). Similar findings have been reported in RAW 264.7 macrophages cultured at 5 kPa (208), and in human diploid fibroblasts upon re-exposure to atmospheric PO_2 following long-term culture at 3 kPa (21). Notably, this phenotype was only observed following long-term adaptation (>5 days) to physiological normoxia (75). Glutathione-based defense systems remained intact, and this alone conferred sufficient protection to maintain cell viability (69). In line with this, basal levels of apoptosis are lower in HUVEC (1) and rat liver sinusoidal endothelial cells (377) cultured at 5 kPa for 3 and 5 days, respectively. We have recently shown that HUVEC cultured at a PO_2 of 5 kPa exhibited significantly less sensitivity to Ca^{2+} overload induced cell death (291), which we attributed this enhanced sarco/endoplasmic reticulum Ca^{2+} ATPase activity sparing the mitochondria from Ca^{2+} overload.

There are conflicting reports on the effects of physiological O_2 levels on endothelial cell proliferation *in vitro*. In the context of angiogenesis, the consensus is that a higher proliferative rates occur in endothelial cells cultured under physiological O_2 levels, and indeed this has been demonstrated in HUVEC (2, 276, 277, 695) and HDMVEC (697). However, we observed a significant reduction in HUVEC proliferation following culture at 5 kPa for more than 5 days (75), in line with the quiescent nature of endothelial cells *in vivo* (133, 244). Moreover, production of extracellular matrix proteins type IV collagen (49, 697), fibronectin, CD105, CD31 and laminin (695) is significantly higher in human dermal microvascular endothelial cells (HDMVEC) and HUVEC cultured at 5 kPa levels. Differentiating between endothelial cell responses to physiological and hypoxic O_2 levels is especially relevant given their unique and highly regulated response to pathological hypoxia. For example, different HUVEC phenotypes are observed when

cultured at a PO_2 of 3 (277) and 5 kPa (2, 75), largely due to increased stabilization of HIF-1α under the lower O_2 level. We have recently demonstrated that the synthesis of nitric oxide (NO) is significantly altered when endothelial cells are cultured at 5 kPa (Figure 10E) (290). Importantly, these processes were still functional, yet under more strict regulatory control by protein phosphatase 2A. This is in stark contrast to the phenotype observed in hypoxic (1 kPa) cells, where stimulation of NO synthesis is largely abolished. In many respects, cells cultured at hypoxic O_2 levels more closely resemble those maintained at room air than those cultured at physiological O_2 levels, further highlighting the importance of this distinction.

5. Circulating and immune cells

As for endothelial and smooth muscle cells, no single O_2 level can be described as normoxic for circulating cells. This does not preclude the study of circulating cells under physiologically relevant O_2 levels *in vitro*, since even the highest O_2 levels encountered *in vivo* (~13 kPa within the pulmonary circulation) are still much lower than atmospheric. Whilst circulating cells are exposed to the full dynamic range of blood physiological O_2 (~5-13 kPa) over a relatively short period of time, the majority of blood at any given time resides in the venous and microvascular compartments (~60-75%) (24, 47), in which PO_2 averages 5 kPa (594). Moreover, most monocytes reside within the spleen at rest (564), where PO_2 ranges from 2-5 kPa in rodents (48, 61, 67, 266) and swine (573), and 7-8 kPa in rabbits (613, 614). Such variance likely reflects the heterogeneous perfusion of the spleen, with pronounced gradients extending away from the splenic artery in mice (61).

Several studies have investigated the effects of isolation and culture of human peripheral blood mononuclear cells (PBMC) under physiological O_2 (as summarised in Table 1-5). Basal proliferation of human CD4+/CD8+ PBMC (16, 17, 315) and murine CD4+ T cells (259) was largely unchanged during culture at a PO_2 of 5 kPa, whereas proliferation stimulated by concanavalin A or CD3/CD28 crosslinking was markedly lower (16, 17). Interestingly, growth stimulated by another lectin, phytohemagglutinin (PHA), was unaffected by culture at 5 kPa (16, 17, 315). Another common observation during culture at 5 kPa is enhanced T-cell differentiation and activation, with increased early expression of the activated-lymphocyte marker CD69 on PBMC (17, 210, 315), activation of PBMC-matured human dendritic cells (171) and murine splenocyte-differentiated T cell CD4:CD8 maturation (61). Indeed, a transcriptional array of human PBMC samples cultured at 5 kPa for 15 days revealed significant upregulation of genes important for lymphocyte biology, whereas expansion at room air highlighted upregulation of gene clusters associated with stress responses, cell death and repair mechanisms (210). Expansion of human PBMC under atmospheric conditions reduces intracellular glutathione (GSH) levels due oxidative stress associated with prolonged hyperoxia. This artefact can be reduced by expansion of PMBC expanded at a PO_2 of 5 kPa (146). Associated with atmospheric-culture induced GSH depletion is

the induction of antioxidant and defense proteins such as superoxide dismutase, catalase (CAT) and glutathione peroxidase-1 (146), resulting in lower basal cytosolic reactive oxygen species (146, 315). One potentially significant consequence of the altered human PBMC phenotype at a PO_2 of 5 kPa was reported by Sahaf and colleagues (499). The HIV-Transactivator of transcription (Tat) protein, previously understood to induce apoptosis in *ex vivo* T-cell preparations, caused rapid proliferation and primed cells for subsequent infection more rapidly than PHA (499). This novel finding highlights the possibility of drawing misinformed conclusions based on *in vitro* experiments performed under hyperoxic conditions. In summary, culture of PBMC and similar cells under physiological PO_2 conditions has significant consequences on cell proliferation, redox status and differentiation capacity. It remains to be investigated whether changes in PO_2 within the physiological range (2-5 kPa), e.g. as would occur when monocytes enter the systemic circulation from the spleen, have functional consequences on their physiology.

Human peripheral blood neutrophils undergo rapid (within 24h) and constitutive apoptosis after isolation and culture under standard culture conditions (PO_2 18-20 kPa). This can be reduced significantly by isolation in a low PO_2 (0-3 kPa) environment (218) and with inhibitors of HIF-1α hydroxylation (628). Moreover, hyperoxia-induced apoptosis was also reduced in peripheral neutrophils isolated from patients with VHL syndrome (627), although this was further reduced by hypoxic incubation, suggesting a role for both HIF-1α-dependent and independent pathways. Despite enhanced survival, neutrophils maintained at a PO_2 of 3 kPa exhibited defective respiratory burst activity and hence killing ability against *S. aureus* infection, seemingly through the limitation of molecular O_2 as a substrate for the generation of ROS (383). This may result from sub-optimal O_2 diffusion/supply capacity *in vitro*, as elegantly demonstrated recently (63), rather than true substrate limitation. The effects of low PO_2 culture on neutrophils has been summarised elsewhere (354, 574) and so will not be discussed in further detail here. Whilst a PO_2 of 3 kPa may be considered ‘sub physiological’ in the general context of this article, one must consider that inflammation can produce localised areas of profound tissue hypoxia (63, 189, 331, 367, 538, 588) and therefore it may be more appropriate to study neutrophil physiology under such conditions, especially if these cells cannot survive at higher PO_2 for more than a few hours. Active neutrophils consume high amounts of O_2 and can actually create or exacerbate a hypoxic environment *in vivo* and *in vitro* (63), which can accelerate the resolution of tissue inflammation.

C. Heart

The heart is second only to the lungs in terms of exposure to O_2, yet in contrast to the lungs is a highly metabolically active tissue with a large O_2 requirement. Although the coronary artery branches directly from the ascending aorta in which blood is at its most oxygenated, measurements of cardioplegic solution PO_2 sampled from numerous coronary artery branches in patients undergoing revascularisation or aortic valve replacement surgery reveal levels of 5

kPa (202). We could not find any measurements of coronary artery PO_2 in healthy humans or experimental animal models, and thus whether these measurements reflect normal physiology or are lower as a result of disease remains unclear. Interestingly, PO_2 values not much lower than these are reported within the coronary sinus in humans (2.7-4.4 kPa (385, 624, 646)), canine (2.9 kPa (69)) and in the great cardiac vein of patients undergoing coronary artery bypass surgery (3.5 kPa (651)). Using a blood-borne phosphorescent probe, Rumsy and colleagues (496) measured the PO_2 distribution in the epicardial microvasculature of the porcine heart prior to and during infarction. An average PO_2 of 2.2 kPa was recorded in non-infarcted tissue, with a gradient observed towards the ischemic core. The coronary microvasculature penetrates the myocardium superficially, and compression of microvessels during systole means perfusion of the deep myocardial tissue is intermittent. As a result, a gradient of PO_2 has been observed between the superficial epicardium, the deep myocardium and individual myocytes, as illustrated in Figure 11. Such levels range from 4.1-6.1 kPa at the epicardial surface (245, 543) to 3.4 kPa in the subepicardium (3 mm depth in the canine heart) (654) and 2-3 kPa in the myocardial tissue (11, 40, 72, 150, 297, 358, 487, 648, 654). Hence, coronary microvascular PO_2 (2.2 kPa (496)) closely matches the average adjacent tissue PO_2, supporting the concept that a significant portion of O_2 diffusion must occur upstream of the capillary bed.

The increase in O_2 between the endocardium and coronary sinus reflects the increasingly acknowledged arteriolar-venular shunting hypothesis of O_2 distribution (328, 594). Generally, excellent corroboration exists in cardiac PO_2 measurements using a variety of techniques, including the use of implantable EPR probes which can be used to monitor cardiac PO_2 with impressive temporal resolution (72, 339, 487) and are stable for at least 112 days *in situ* (297), though these typically do not offer great spatial resolution. Anatomical complexity in O_2 delivery is compounded by the extremely high O_2 consumption rate of cardiomyocytes (see Section IV-C), resulting in very low intracellular PO_2 (1-1.6 kPa (84, 252)), though this low O_2 does not limit aerobic respiration in these cells (84). Myoglobin, present within the cytosol of skeletal and cardiomyocytes, has a greater affinity for O_2 than hemoglobin (P50 of ~0.3 vs ~3.5 kPa (12)) and is thought to facilitate O_2 delivery to respiring mitochondria (567, 568). It may do this simply by providing a store of O_2 within the cell, that can be made available during periods of increased activity, or perhaps by ‘removing’ dissolved O_2 from the cytosol thereby increasing the concentration gradient from the blood to cell in a process of facilitated diffusion (90, 234, 281, 657, 658). Hence. actual O_2 content within a cardiomyocyte *in vitro* will be higher than the detected dissolved PO_2, complicating interpretations of relative monolayer oxygenation.

Stem cells derived from human myocardial biopsies cultured at a PO_2 of 5 kPa exhibit greater chromosomal stability and fewer karyotypic defects over 1-2 months of culture than their room air

counterparts (343). Moreover, a greater yield was achieved per biopsy, with a higher reparative capacity when subsequently transplanted into a mouse infarcted heart (342). Interestingly, Puente et al. (471) recently demonstrated that the dramatic rise in cardiac tissue PO_2 in the fetal heart at birth induces growth arrest through oxidative damage. This elegant study may explain the enhanced proliferative and reparative potential of cardiac stem cells under physiological O_2 levels, especially when one considers that the true intracellular O_2 level in these cells may be considerably lower than 5 kPa.

Murine cardiac fibroblasts cultured at a PO_2 of 3 kPa were also found to proliferate more rapidly, which was attributed to reduced expression of p21 and a less oxidised environment (494). However, since intracellular PO_2 in these cells are likely to be considerably lower than the ambient (and physiological) 3 kPa, these results more accurately represent a hypoxic phenotype acknowledged by the authors. In line with this, human cardiac fibroblasts cultured under perfused conditions at a PO_2 of 5 kPa exhibit decreased growth relatively to room air and hypoxic (1 kPa) cultures (600), suggesting hypoxia and hyperoxia, but not physiological normoxia, are potent triggers for fibroblast proliferation *in vitro*. In this recent study, a clear delineation between the cellular response to normoxia (5 kPa) and hypoxia (1 kPa) is demonstrated, with hypoxia strongly associated with markers of pathological cardiac remodeling (600). However, cells were subjected to altered ambient PO_2 for only 24 h in this study, and hence may not represent truly 'adapted' cells. Rat neonatal cardiomyocytes isolated and maintained at a PO_2 of 5 kPa release higher concentrations of adenosine but rapidly release the vasoconstrictive angiotensin II when exposed to higher O_2 levels (655). Although limited, these studies collectively demonstrate that culture of cardiac-derived cells under physiological O_2 levels robustly alters their phenotype, with significant therapeutic implications.

D. Brain

The brain receives blood from mainly the common carotid artery and to a lesser extent the vertebral artery, both of which branch directly from the ascending aortic arch. In line with microvascular O_2 distribution outlined above, oxygen levels of ~8 kPa have been measured in rat (70, 527) and mouse (501) pial arterioles. Moreover, a PO_2 of ~3.5 kPa in the pial parenchyma (at depth of ~50μm) (390, 527), indicate a significant loss of O_2 across the pial arteriolar wall (527). Similar to other major organs, the cerebral vasculature extends superficially throughout the brain with fewer vessels penetrating the inner layers of the cortex. Accordingly, PO_2 within the cortex decrease proportional to the depth: from ~5 kPa the superficial cortex (70, 111, 247, 248, 351, 688) to ~3 kPa in the deep white matter (70, 111, 390, 501, 544), as illustrated in Figure 12. This gradient further extends into the deeper regions associated with CNS function (hypothalamus, hippocampus, midbrain). Surprisingly, PO_2 levels within these regions have been measured as low as 0.5 kPa (0.5-1 kPa midbrain (70), 0.4 kPa pons (70), 1.5-2

kPa hypothalamus (70), 0.8-3.5 kPa caudate nucleus (525)). However, Lyons and colleagues (364) have challenged this concept recently. Using 2-photon PQM (phosphorescence quenching method, Section II-C), they observed no change in intravascular PO_2 in either arterioles or venules of the somatosensory cortex with increasing depth from the surface (364). These authors proposed that anesthesia and associated stressful handling during the experiment setup may artificially alter brain activity and hence tissue PO_2 (364). Prior measurements were made using the surgical insertion of a microelectrode, and therefore revisiting brain tissue PO_2 levels using 2-photon light microscopy could provide further insights (501, 502). Indeed, Lecoq and colleagues (328) were able to measure neuronal PO_2 in the mouse olfactory bulb during odour stimulation, in which average microvascular PO_2 increased from ~6 kPa to 7.5 kPa, simultaneous with similar increases in red blood cell velocity and intracellular Ca^{2+} (328). Notably, neuropil PO_2 was substantially lower than the surrounding microvasculature (~3 kPa), yet still increased upon odour stimulation. In subsequent studies, a mean interstitial PO_2 of 3-4 kPa was found in the murine olfactory bulb and somatosensory cortex (364, 445), consistent with previous studies using implanted ESR probes (125, 350, 439). Changes in brain oxygenation during stimulation or disease have become the focus of increased experimental and clinical studies, the former spurred on by the development of BOLD-MRI (see Section II-D). Whilst blood-oxygen level dependent functional MRI imaging has established clinically that changes in blood saturation correlate with changes in neuronal activity within the predicted area (as reviewed (164)), this technique cannot be used to quantify absolute changes in PO_2 in the brain. However, several recent studies using microelectrode and phosphorescence-quenching techniques have demonstrated quantitative changes in brain tissue PO_2 (either in the microvasculature or surrounding neurons) upon sensory stimulation (256, 328, 445, 692). In these studies, stimulation of neuronal activity was associated with a transient reduction in tissue PO_2, likely representing localized increases in O_2 consumption, followed by increases in local blood PO_2 and flow, confirming similar observations using fMRI.

Neurons isolated from rat striatum (578) and cortex (698), human fetal neurons and the neuronal cell line SH-SY5Y (617), as well as mouse astrocytes (95), have all been studied under physiological normoxia (defined by authors as an ambient PO_2 of 3-5 kPa). A common phenotypic change was observed in all these cell types following normoxic culture, characterised by a hyperpolarized mitochondrial membrane potential, reduced reactive oxygen species generation and greater survival during prolonged culture. The latter two features may result from enhanced activity of manganese superoxide dismutase under low O_2 (617). The most significant impact to be uncovered following culture under normoxia is on neuronal mitochondria. In addition to mitochondrial hyperpolarization (578, 698), more dense mitochondrial networks have been observed (578). Moreover, a proteomic screen of SH-SY5Y cells maintained at a PO_2 of 5 kPa revealed significant induction of mitochondrial complex I proteins, most notably NADH:ubiquinone

oxidoreductase subunit 3, which was associated with greater sensitivity to the complex I inhibitor rotenone (617).

Neuronal stem cells (NSC), otherwise known as neuronal precursor cells, are adult stem cells which differentiate into neurons, astrocytes and glial within the CNS. Neurogenesis generally occurs in regions of the inner brain, such as the hippocampus and dentate gyrus (135), and as such occurs within a physiological PO_2 range of 0.5-3 kPa (see Figure 12). Neuronal stem cells from the embryo (554), sciatic nerve (409), cortex (555), midbrain (561) and ganglionic eminence (255, 258) all exhibit higher proliferation, survival and differentiation potential when cultured at 3 kPa. These effects were associated with enhanced HIF-2α stabilization and downstream protein expression (258, 554), resulting in an increased transplantation potential following *in vitro* expansion (554, 555). NSC from whole mouse brain homogenates cultured at 5 kPa also exhibit a higher differentiation potential associated with greater mitochondrial oxidative phosphorylation and reduced production of reactive oxygen species (563). Though informative, this latter study makes no justification for the level of O_2 used, instead opting for the arbitrary use of 5 kPa as physiological normoxia. Whilst not necessarily incorrect, the numerous studies listed above suggest that NSC may rarely experience a PO_2 of 5 kPa. Nonetheless, these and other studies have led many to propose a causal relationship between O_2 concentration and stem cell differentiation potential (625), with numerous cell types displaying enhanced differentiation potential under lower O_2 levels. This is corroborated by the anatomical topology of neurogenesis, which occurs predominantly in the brain regions containing lower O_2 (Figure 12).

E. Liver

The liver is a highly vascularized and metabolically active tissue, and as such is relatively well oxygenated as illustrated in Figure 13. Blood entering the hepatic artery is fully saturated and rich in O_2 (human 12.2 kPa (526)). Processed blood exiting the hepatic vein reportedly has a PO_2 between 4-6.5 kPa (526, 598, 660), whilst blood entering through the portal vein is at ~6.5 kPa (526, 598, 660). This dramatic gradient likely reflects the mixing of hepatic arterial blood with venous portal vein blood (at a ratio of 1:3) before entering the hepatic triad, as well as a high metabolic demand of the underlying tissue. Indeed, blood PO_2 within the portal triad is already reduced compared to that of the hepatic artery (8.7 kPa (660). This level is further reduced within the liver parenchyma, where a PO_2 of between 4-7 kPa has been reported (54, 55, 103, 312, 327, 546, 608). Using the oxygen-sensitive EPR probes India ink and lithium phthalocyanine, Jiang et al. (275) were able to measure PO_2 localized to Kupffer cells and whole liver PO_2, respectively, and demonstrated that Kupffer cells experience a lower PO_2 than the average liver parenchyma (2 vs 3.2 kPa). These values are lower than expected within the liver, possibly reflecting differences in experimental technique. The gradient between the portal triad and vein creates a functional polarization of the underlying hepatocytes, wherein nitrogen metabolism

and albumin secretion are proportional to cell surface O_2 level (37). This allows a similar effluent composition under conditions of flow reversal. Clearly, although a significant loss of O_2 occurs during passage through the liver, this organ is still extremely well oxygenated in relation to other similarly active tissues such as the heart or brain (377).

Studies *in vitro* have provided some explanation for the abnormally high oxygen levels in liver tissue. In contrast to the majority of cell types, hepatocytes isolated from rat livers and maintained under low O_2 (4-10 kPa) for the duration of culture (3-9 days) responded adversely. This included reduced viability (37, 345, 669) and diminished liver-specific gene expression (299, 345) and functions such as nitrogen metabolism and albumin excretion (37, 669). Moreover, several reports have highlighted the enhanced infectivity of liver-associated pathogens such as Hepatitis C and *Plasmodium berghei* (during liver stage malaria) in hepatocytes under low O_2 conditions *in vitro* (421, 612). Interestingly, acetaminophen toxicity was less pronounced in mouse hepatocytes cultured at a PO_2 of 5 kPa relative to room air (668), although significantly higher basal LDH release was observed in these cells. Part of this adverse response can be attributed to the very high O_2 consumption rate of primary hepatocytes which, during static cell culture *in vitro*, can create an extensive O_2 gradient between air and cytosol (see Section IV-C) (75, 671). In this context, culture under previously regarded physiological PO_2 (4-10 kPa) may actually expose the cell monolayer to a PO_2 as low as 0.5 kPa (421). In corroboration, exposure to relatively high (but still lower than ambient) PO_2 (13-17 kPa) proved beneficial for cell yield (345, 368) and the response to phenobarbital challenge (299). HepG3 and Hep3B cells cultured at 8 kPa for up to 30 days had higher intracellular, but not mitochondrial, reactive oxygen species generation with severely reduced intracellular GSH levels (587). Despite this, SOD activity and expression were significantly higher in these cells, and they exhibited greater resistance to oxidative challenge. These studies in hepatocytes emphasize the need to understand monolayer oxygenation more closely. Ambient PO_2 during *in vitro* culture must be accurately tailored to the cell type to avoid exposing high O_2-demanding cells to cellular hypoxia (204).

F. Kidneys

The kidneys comprise only 1% of total body mass, yet filter ~20% of cardiac output (4.2 mL/min/g tissue), and as such are disproportionately perfused compared to the other organs. Indeed, renal cortical blood flow is the highest recorded per g tissue within the body (386), and the metabolic requirements are relatively low under normal conditions, yet increase linearly with glomerular filtration rate, renal blood flow and Na^+ transport (479, 642). Considering such hyperperfusion, renal tissue should be extremely well oxygenated as with other well-perfused organs such as the liver (see above). Counter-intuitively however, renal cortical PO_2 has been measured at 4.7 kPa in the dog (28), 5-7 kPa in the rat (50, 51, 203, 334, 349, 362, 519, 642), 6 kPa in the pig and 4-9.5 kPa in human (411, 541). When the measuring electrode is advanced beyond ~3mm (in the rat

kidney), a sharp reduction in tissue PO_2 is observed corresponding to entry into the medulla (see Figure 14), where O_2 levels are between 1 kPa in the dog (28), 1.3-4.2 kPa in the rat (50, 51, 203, 334, 349, 362, 519, 642), 3.3 kPa in the pig (693) and 2 kPa in human (693). In elegant work by Lübbers and Baumgärtl, a large heterogeneity in cortical PO_2 was observed whereby some regions experienced near-arterial PO_2 values (~11 kPa) (334, 362). Such heterogeneity was not apparent within the medulla, prompting these authors to conclude that the highly vascularized anatomy of the cortex results in the formation of large intra-renal PO_2 gradients. Using ultramicroelectrodes (diameter 3-5µm), Welch and colleagues achieved measurements of PO_2 in the nephrons of rats (642). In line with previous data, a significant drop in PO_2 was reported between the renal artery and efferent arterioles (10.7 to 6 kPa), and tubular PO_2 remained around 6 kPa before increasing within the renal vein to 6.7 kPa (see Figure 14). Kidney PO_2 values determined by Clark electrode penetration were corroborated by recent measurements using BOLD MRI (395, 451, 693), confirming the validity of both techniques. Notably, measurement of kidney PO_2 using a dual laser-Doppler blood flow/O_2 electrode technique consistently demonstrated lower cortical PO_2 values than the corresponding single O_2 electrode (1.3 (434) and 2.1 (463) vs 5.3-6.7 kPa). This disparity was attributed to the larger diameter O_2 electrode required in the dual electrode array compared to that used in tissue penetration studies (350-500µm vs <20µm), potentially resulting in damage to the delicate kidney architecture (434).

The substantial drop in PO_2 between the renal artery and cortical tissue prompted many to postulate the existence of a pre-glomerular O_2 shunt (28, 334, 338). This was confirmed by Schurek and colleagues who demonstrated that inspiration of gas at 100 kPa PO_2 led to minimal changes in glomerular PO_2 (5.6 to 9 kPa) despite a robust increase in systemic PO_2 (12 to 74.6 kPa) (519). The close anatomical relationship between interlobular arteries and veins, and the absence of surrounding capillaries (141, 422), is thought to be responsible for such a shunt in line with the arterio-venous O_2 transfer theory proposed by Duling and Berne (123). Indeed, renal vein PO_2 is unusually high compared to systemic venous PO_2 (measured at 6.5 and 7.8 kPa in the rat (642) and dog (201), respectively, vs ~5 kPa), further corroborating renal arterio-venous O_2 transfer. Moreover, whilst whole kidney O_2 consumption is second only to the heart (10 vs 15 ml/min/g), renal blood flow far exceeds myocardial blood flow (750 vs 250 ml/min) and thus renal O_2 extraction is only ~10% vs 55% in the heart (452, 479, 485, 687). Taken together, these observations led O'Connor and colleagues to hypothesize that renal tissue is maintained at a low PO_2 level relative to blood flow to prevent over-oxygenation and excessive oxidative stress (433). Unfortunately, the side-effect of this proposed adaptation is that segments of the papillae within the medulla (in particular the S3 proximal tubule and medullary thick ascending limb) are exquisitely sensitivity to reductions in blood flow, and are the first sites damaged during acute renal ischemia (46, 130).

Considering the prominent role of hypoxia-inducible factor signaling in the kidney (209) and its sensitivity to low oxygen (167, 398), relatively little has been published on the long-term culture of kidney-derived cells under physiological normoxia. This may, in part, be complicated by the significant differences in what may constitute normoxia in this tissue, with medullary PO_2 values often 4-5 kPa O_2 lower than corresponding cortical tissue (see above). Much like the gastrointestinal tract (see Section VI-G), parts of the renal medulla may exist in a state of 'physiological hypoxia'. In intestinal epithelia, cellular phenotypes characterised at a PO_2 of 1 kPa appear a better reflection of their *in vivo* counterparts than those cultured under room air. However, exposure of renal tubular epithelial cells to hypoxia (1-3 kPa) results in robust HIF downstream target protein (VEGF and EPO) induction (129, 209, 337, 417), phenotypes not observed in their counterparts *in vivo* (491). This discrepancy may reflect the artifactual baseline cellular PO_2 prior to hypoxic exposure (room air, PO_2 ~18 kPa), potentially exacerbating the subsequent hypoxic response. It would be interesting to explore whether small reductions in cellular PO_2 around physiological normoxic values (from 1.5/2 to 1 kPa) can elicit more physiologically relevant responses in these cells. Such experiments may identify additional, more subtle roles for the HIF in renal cells, and in doing so shed further light on the renal cell specificity of von Hippel-Lindau syndrome (29, 419, 503).

G. The digestive system

The discovery of anaerobic bacterium resident within the intestinal lumen led to the concept of 'physiological hypoxia' within the adjacent intestinal tissue (696), with important implications for our understanding of host-microbiome interactions (292). This section aims to review the literature, with an overview of digestive system PO_2 and discussion of the importance of considering the O_2 environment during culture of cells derived from the digestive tract *in vitro*. The digestive tract is characterised by an adventitial (serosa) layer, a muscular middle layer and an epithelial (mucosal) layer. Large arterioles run longitudinally along the serosa and the microvasculature (mesentery) penetrating towards the mucosal layer. Although the digestive tract receives a high proportion of blood flow, only 25% of this perfuses the mucosal and muscular layers at rest (81, 191). Measurements of digestive tract PO_2 have been divided into luminal, mucosal and serosal tissue, and the gradient between these values discussed where evident.

1. Stomach

The stomach, similar to the lungs and skin, can come into direct contact with atmospheric levels of O_2. As a result, PO_2 measurements at the submucosal aspect of the gastric fundus using micro-electrodes are in the range of 6-10.2 kPa in humans undergoing eosophagectomy (94, 264, 265, 529) and 7.6 kPa in experimental canine models (318), with similar values (8.4 kPa) reported in canine serosa (353). Using activated charcoal as an EPR probe (see Section II-D), He and colleagues (228) determined a luminal PO_2 of 7.7 kPa at the level

of the fundus/pylorus. Since PO_2 values are roughly equal across the gastric wall (lumen>mucosa>serosa, Figure 15C), and such a level is not dissimilar from what might be expected in the arteriolar circulation (see Section VI-B-2 and Figure 9), we might conclude neither the contents of the stomach or the cells of the gastric wall represent a significant source of O_2 consumption, at least under basal conditions.

2. Intestines

As illustrated in Figure 15A, a longitudinal gradient in luminal PO_2 is observed during passage along the gastrointestinal tract. From a luminal PO_2 of 7.7 kPa in the stomach (228), luminal PO_2 in the small intestine decreases to between 4.2-4.6 kPa (312), and decreases even further to 0.4-1.5 kPa in the large intestine (9, 228, 294, 346). Importantly, the techniques used to obtain such data, including EPR and 2-photon phosphorescent quenching, are non-invasive and therefore avoid contaminating the bowel environment with atmospheric air and preserve the integrity of the bowel wall. A similar longitudinal gradient is observed in mucosal PO_2 (Figure 15A); from 6-10 kPa in the stomach to values of 3.5 kPa (rat (42)), 5.8 kPa (dog (353)), 2.6-5 kPa (pig (212, 213, 305, 429, 540, 611)) and 4.5-4.8 kPa (humans (529)) in the small intestine and values less than 1 kPa in the colon (9, 294). A similar longitudinal gradient is present, albeit less pronounced, at the serosal aspect (Figure 15A). Small intestine serosal PO_2 is 5.6 kPa (rabbit (163)), 7.2-8.4 kPa (pig (157, 213, 429, 540)) and 8.1 kPa in humans (570). In the large intestine, PO_2 at the serosal layer is reported at 4.6 kPa (rabbit, (163)), 3.7-4.5 kPa (pig, corrected for FiO_2 0.21, (301, 388)), 5.3 kPa (mouse, (9)) and 3.9-5.5 kPa (human, (412, 529, 530)).

From such data, it is clear that O_2 levels show a general decline along the length of the gastrointestinal tract, being highest in the stomach and lowest in the colon. It is perhaps of more interest to note that the radial gradient across the intestinal wall (lumen to serosa) does not behave proportionally. No detectable difference is observed between the lumen and serosa of the stomach, whereas the lumen of the large intestine is ~5 kPa lower than the serosal layer (Figure 15A). This radial gradient has been demonstrated directly in the small intestine using the penetrating microelectrode technique (42, 212, 429), by 2-photon PQM (540), and by pimonidazole staining (157), and also in the colon (294) (representative immunohistochemical images from the latter studies are shown in Figure 15B). Data from the study by Bohlen indicates a difference of as much as 2.3 kPa O_2 between the apex and base of a villus (42), whilst a difference of 4.6-9 kPa is reported between the mucosa and serosa (212, 429, 540). This is corroborated by a gradient in pimonidazole staining along the villus length (157, 294). It was originally proposed that this gradient occurs due to an arteriolar-venular O_2 shunt at the base of the villus, where vessels reside in close proximity to each other (42). However, the finding that this gradient is abolished by antibiotic treatment and is absent in germ-free mice (294) would suggest the O_2 consumption by the gut microbiome is wholly responsible. Upon reflection, this is logical given that the bacterial

population increases along the gastrointestinal tract, whereas there is no evidence to suggest the proposed arteriolar-venular shunt shows similar topographical changes.

The extremely low physiological PO_2 experienced by intestinal epithelium *in vivo* creates an interesting scenario whereby there is probably no real distinction between hypoxia and normoxia, but rather a state of 'physiological hypoxia' exists in the intestinal mucosa (92, 696). Hence those pathways traditionally important in coordinating a cellular response to pathological hypoxia, such as the HIFs (see Section V-A-1), may have more importance in defining the phenotype of intestinal epithelia *in vivo*. Indeed, a healthy gut epithelium respiring on their preferred substrate butyrate exhibits profound HIF response element (HRE)-luciferase activity and HIF-1α stabilization (64, 294), indicating a persistent activation of the pathway. This constitutive activity was found to be essential for the production of defensins, key endogenous antimicrobial peptides secreted by the intestinal epithelium and accumulated within the mucus layer (293), which itself is regulated by HIF-1α at a number of levels (170, 359). Moreover, expression of the multidrug-resistance protein 1 (P-glycoprotein) was also found to be dependent on constitutive HIF-1α activity in the intestine (93). HIF activity is also crucial in maintaining intestinal barrier integrity, with key junctional proteins such as claudin-1 and creatine kinases M and B regulated by HIF-1 and HIF-2, respectively (180, 498). Notably, a key role for constitutive HIF-2α activity in maintaining iron absorption has also been identified in the mouse duodenum, the epithelium of which also exhibits strong basal pimonidazole staining (379). Hence, the state of physiological hypoxia is important in maintaining host defence, barrier integrity and iron absorption throughout the gastrointestinal tract.

Importantly in the context of replicating normoxia *in vitro*, experiments in which intestinal epithelial cell lines (Caco-2 and T84) are exposed to physiological normoxia (PO_2 1-2 kPa) generally corroborate such observations (see Table 1-6 for summary). Notably, although few studies have extended the period of normoxic culture beyond 24 h, a number did demonstrate a stable phenotype after up to 4 days of normoxia (93, 566). A point worth discussing is the apparent discrepancy between the effects of low O_2 exposure on transepithelial resistance (TER) *in vitro* and *in vivo*. Exposure of Caco-2 and T84 cells to a PO_2 of 1-2 kPa induces no change or increases in TER *in vitro* (170, 284, 566), a characteristic unique to intestinal epithelial cells (170). In contrast, intestinal permeability is consistently decreased in mice breathing low O_2 (8 kPa) (170, 284, 408, 566). Aside from technical differences, there are two possible explanations for these observations. Firstly, hypoxic exposure does not alter TER *in vitro* relative to room air cultured cells, a condition wholly artificial in the context of physiology. Moreover, if mucosal PO_2 is normally physiologically hypoxic (see above) then a reduction in inspiratory PO_2 (from 20.9 to 8 kPa) may create a genuinely pathologically hypoxic/near anoxic environment within the mucosal intestinal epithelium. Similar conditions are observed during inflammatory bowel disease and

ulcerative colitis (64, 284), conditions associated with increased intestinal permeability. Thus fundamental differences in the context and degree of 'hypoxia' under these experimental conditions make comparisons difficult to interpret. Secondly, whole body hypoxia undoubtedly has effects beyond the intestines, and it is therefore difficult to isolate the actions of any paracrine mediators released by other tissues also responding to reduced systemic arterial PO_2. Notably, work by Zeitouni and colleagues (689, 690), using O_2-sensitive chips immobilized on the bottom of standard culture plates, indicates that exposure of Caco-2 cells to an ambient PO_2 of ~1 kPa results in a near anoxic medium PO_2 after 6 days (690). However, it should be noted that the authors used 1 mL of medium in a 24-well plate, double the recommended volume for such dishes. Using the example discussed in a previous section (Section IV-C), these conditions would generate a value for *d* double that commonly used (0.2 mm), facilitating 'self-inflicted hypoxia' in this scenario. Ideally, the correct *in vitro* experiment to accompany *in vivo* hypoxia models would be exposure of intestinal epithelial cells to very low (PO_2 ~0-0.5 kPa) O_2 from a baseline of 1-3 kPa to more accurately reflect conditions generated *in vivo*.

3. The Pancreas

Interest in pancreatic PO_2 distribution arose largely out of the need to understand the oxygenation of endogenous islets in order to match this when implanted elsewhere. Using a microelectrode, Harper and colleagues (226) reported a PO_2 of 3.3 kPa in the acinar/exocrine region of the exteriorised rat pancreas. This was corroborated by more detailed measurements by Carlsson and colleagues (66, 67), who reported a gradient from 4.6 to 2.7 kPa O_2 between the surface and interior of the exocrine pancreas. Kinnala and colleagues used a technique termed suffusion tonometry to monitor whole organ PO_2 in the porcine pancreas. Briefly, a silicone tube is implanted into the tissue and equilibrated with anoxic saline. After a subsequent period of equilibration with the tissue, fluid is extracted from the tube and PO_2 determined using a standard electrode. Using this technique, they reported pancreatic PO_2 values of 5.3 (302) and 5.6 (303), thus reasonably matching reported values using microelectrode techniques. The Islets of Langerhans are important anatomical features of the pancreas wherein insulin and glucagon are synthesized and secreted. These tiny collections of cells constitute only 1-2% of total pancreatic tissue, yet receive 10-15% of organ blood flow (268). This extensive perfusion is reflected in a PO_2 level considerably higher than the surrounding parenchyma, between 5-6 kPa in the rat (66, 67).

The high rates of O_2 consumption in pancreatic β cells has presented a unique problem during their isolation, culture and implantation. Isolated β cells typically exist in culture as suspended spheroids, and pimonidazole staining illustrates that such conditions produced severe hypoxia (113, 438) which is proportional to medium PO_2 and islet size (310). This also extends to monolayer cultures of the pancreatic cell line MIN6, in which intracellular PO_2 (based on pimonidazole staining) was far below ambient (511). Moreover, exogenously implanted islets

rapidly develop hypoxia regardless of their anatomical location (66, 67), reflecting an inability of neo-vascularisation to counteract islet O_2 consumption. Attempts to address this problem have included the use of permeably culture supports, such as silicone membranes (18, 304) perfluorocarbon (PFC)-based culture dishes (166) or O_2 leaching materials (96), as well as culture under hyperoxic ambient PO_2 (50 kPa) (309). Using a PFC-PDMS construct, Fraker and colleagues elegantly demonstrated that the combination of high substrate solubility and lower ambient PO_2 (~12.6 kPa) produced the greatest volume of physiologically oxygenated (predicted pericellular PO_2 of ~5.5 kPa) β-cells and almost no anoxic cells compared to culture under room air on traditional plastic ware (166). This translated into greater β-cell viability and function following isolation and a trend towards greater *in vivo* survivability once transplanted.

H. Bone and cartilage

1. Bone and bone marrow

Typical bone consists of an outer cortical/compact layer, a spongy/cancellous inner layer and the medulla, containing the bone marrow (Figure 16A). The outer mineralized layers are sparsely populated with cells and are effectively avascular, and the only vessels found within this layer are the superficial periosteal arteries and penetrating nutrient arteries perfusing the bone marrow. As such, mineralized bone tissue exists at relatively low PO_2. In the only known publication measuring bone extravascular PO_2, Brighton and Heppenstall reported a steep gradient in PO_2 between the diaphyseal and metaphyseal regions (14.5 to 2.6 kPa) of the rabbit proximal tibial epiphyses (52). Since the reported PO_2 in the diaphyseal region is beyond the physiological arterial range (12-13 kPa), the accuracy of these values necessitates further study. The concept of a steep gradient between regions of mineralized bone has been predicted mathematically (685). More recently, measurement of PO_2 distribution in the mouse tibia was partially accomplished using implantable LiPc crystal EPR probes (see Section II-D) (360, 361). PO_2 at the periosteum of normal tibia was 6 kPa (360), whereas localized PO_2 within the bone upon fracturing decreased significantly to ~1.5 kPa immediately after injury (361). Subsequent angiogenesis and healing resulted in a robust increase in bone PO_2 (from 1.5 to ~7 kPa), indicating that neovascularization of damaged bone can restore normal O_2 distribution (361). Notably, periosteal PO_2 values reported by Lu and colleagues match closely the intravascular PO_2 values in microvessels of the periosteum (6.6 kPa (552)), suggesting a limited radial gradient in this tissue which is consistent with the sparsely populated nature of mineralized bone.

The isolation and maintenance of human stem cells under low O_2 has generated promising advances in their clinical application. Whilst effects of culture under physiological O_2 levels on partially differentiated precursor stem cells have already been discussed under the auspice of their respective tissue origin, bone marrow is a primary source of adult pluripotent stem cells. Furthermore, this tissue is also the source of hemopoietic stem cells which give rise to all

circulating blood cells. As such, a detailed understanding of their physiological environment is of clinical relevance. PO_2 has been determined in human sternum (5.4 kPa (192)), and iliac crest marrow (6.8 (262), 7.2 (227), 6.1 kPa (154)), canine femoral medulla (6.4 kPa (308)) and mouse femoral marrow (~4 kPa (71)). These measurements were largely obtained from blood-gas analysis following the aspiration of marrow. Recently, detailed *in situ* measurements of marrow O_2 levels in living mice have been obtained using the more advanced 2PLM method (see Section C) (552), with impressive resolution. Spencer and colleagues (502) reported an intravascular PO_2 gradient between vessels in the periosteum (6.6 kPa), cortical bone (4.2 kPa) and marrow (3 kPa). By comparing intravascular and extravascular PO_2 within the bone marrow, these authors identified a significant radial gradient (~1.5 kPa) in these vessels which could by abolished by sub-lethal irradiation or chemotherapy. Indeed, localized bone marrow hypoxia was associated with large clusters of cells within the marrow, indicating that O_2 consumption maintains a low PO_2 environment in the bone marrow.

Whilst elegant and informative, the study by Spencer and colleagues reports bone marrow PO_2 values considerably lower than values reported by numerous other laboratories (~2 vs ~5 kPa). Tissue trauma associated with bone marrow aspiration or microelectrode penetration may lead to artificially high marrow O_2 level estimations and hence the non-invasive measurements by Spencer et al. may be physiologically representative. Species differences are an unlikely explanation, since Ceradini et al. (71) reported a bone marrow PO_2 of ~4 kPa in the mouse femoral marrow. Instead, regional differences in bone PO_2 may provide some clarification, with Spencer et al. opting to study the calvaria (skull cap) in contrast to the femur, sternum or iliac crest. Similarly, PO_2 distribution in the brain microvasculature differs subtly from other microvascular beds (Figure 9).

2. Cartilage and synovial fluid

Many bones are connected via a synovial joint consisting of a fluid filled synovial cavity and articular cartilage lining the epiphyses of the bone (Figure 16A). In addition to providing mechanical support during movement, synovial fluid is also crucial in supplying nutrients, including O_2, to the articular cartilage, which itself is completely avascular. Chondrocyte physiology is strongly influenced by O_2 tension, during development and in adult life (513), and increases in O_2 consumption are observed during rheumatoid arthritis-associated inflammation (332). For these reasons, understanding PO_2 distribution within the synovial joint is of great interest. Of the studies reporting synovial PO_2 values, only two investigated synovial fluid PO_2 in healthy tissue. Ferrell and Najafipour (1992) reported a mean PO_2 of 6.4 kPa in the rabbit knee (152). Using microelectrode penetration, these authors also demonstrated a gradient in PO_2 between the superficial synovial membrane and articular cartilage, at which point mean PO_2 reached ~1.5 kPa, a value corroborated in human septal cartilage more recently (482). Although such PO_2 distribution traces are commonly reported in numerous tissues, the study of a synovial joint under immobile

anesthetised conditions may create an artifactual gradient which would not otherwise be present in a mobile joint (152). Indeed, Etherington and colleagues (137) reported a transient increase in knee synovial PO_2 upon mobilisation of the joint. A number of studies have reported synovial fluid PO_2 in the human arthritic knee, with values ranging from 2.6 (38), 3.5 (363), 3.7 (143), 4.1 (589), 5.7 (363) to 7.3 kPa (484). Inflammatory arthritis was also associated with a decline in knee synovial PO_2 in rabbits (416) and mice (137). In general, synovial PO_2 correlated negatively with the severity of inflammation (589), with relationships to blood flow (416) and white blood cell count (484) observed. Furthermore, patients with osteoarthritis had significantly higher synovial fluid PO_2 than those with rheumatoid arthritis (363). Hence it is likely that the inflamed nature of the arthritic joint produces a pathophysiologically 'hypoxic' environment relative to the normal joint, and thus PO_2 values derived from the study of such joints likely underestimate physiological normoxia.

3. Resident bone cells

As described in Sections H-1 and H-2, the avascular nature of mineralised bone and cartilage creates a physiological milieu containing relatively little O_2 (~1.5 kPa in deep cortical bone and articular cartilage) for resident cells. Table 1-7 provides an overview of studies conducted to investigate the effects of culture at physiological normoxia on bone cell physiology. Long-term culture (>72 h) of osteoblasts at the lower end of this physiological range (PO_2 2 kPa) is associated with a significant reduction in mineralization (Ca^{2+} deposition as measured by Alizarin red staining) and alkaline phosphatase activity compared to room air cultures (329, 423, 435, 601, 686). This was reportedly due to a delayed (601) or reduced (423) expression of the osteocyte markers osteocalcin and procollagen lysine dioxygenase, and was associated with a reduced ability to synthesize and deposit collagen (435). Indeed, microarray analysis of human osteoblastic cells (SV-HFO) cultured at 2 kPa for up to 3 weeks revealed the greatest impact of low O_2 on the mineralization phase of differentiation (424). However, culture under an ambient PO_2 of 2 kPa would result in a much lower intracellular PO_2 and hence this phenotype is likely representative of a reactive, hypoxic cell rather than the physiological norm. This hypothesis is corroborated by the observation that pre-osteoblastic cell lines (MC3T3-E1 and MLOA5) cultured long-term at a PO_2 of 2 kPa exhibit marked HIF-1α stabilization (686), pimonidazole staining and HRE luciferase activity (329), indicating a classical hypoxic phenotype. In contrast, paired cultures maintained at a PO_2 of 5 kPa showed no such HIF-1α activity (329), consistent with our findings in human endothelial cells maintained >5 days under similar conditions (see Figure 10). Under this more physiologically relevant PO_2 (accommodating for monolayer consumption), MC3T3-E1 cells exhibited earlier and more robust mineralization and alkaline phosphatase activity, which was associated with increased expression of the osteocytic markers osteocalcin, Dmp1, Mepe and Fgf23 (241). Similar findings were reported in murine calvarial organ cultures maintained under 5 kPa O_2 (241). These findings suggest that higher O_2 levels may stimulate osteocyte maturation and mineral bone formation *in vivo*, supported by the knowledge that the highest intraosseal PO_2

values (6-7 kPa) recorded are in the periosteal layers of diaphyseal bone (52, 552). Thus, the appropriate intracellular O_2 level *in vitro* should be defined based on the differentiation state to be studied.

4. Chondrocytes

Grimshaw and Mason (2000) (196) attributed the unusual ability of chondrocytes to withstand severe hypoxia to a very low mitochondrial mass *in vivo* (53) and low O_2 consumption rates *in vivo* and *in vitro* (240). As chondrocytes experience very low O_2 levels *in vivo* (~1.5 kPa, see Figure 16A), O_2 conservation may be necessary. There are numerous studies of chondrocytes during culture under normoxic conditions *in vitro*, as summarised in Table 1-8. Such studies have largely focused on culture techniques to reverse the de-differentiation of primary chondrocytes that occurs following isolation and monolayer culture *in vitro*. De-differentiation is characterised by a switch in collagen production from type II to type I (34), with concomitant downregulation of key chondrocyte markers aggregan and SOX9. Whilst very low O_2 (<1.5 kPa) levels exist in deep articular cartilage, new cartilage is formed at the superficial layer adjacent to the synovial fluid where PO_2 values are closer to 5 kPa (see Figure 16A). When cultured on alginate beads at a PO_2 of 5 kPa for 2-4 weeks, the phenomenon of redifferentiation is accelerated and enhanced in human (287, 414, 518), bovine (117, 118, 239, 415), mouse (242) and rabbit (233) chondrocytes. In addition to the expression of *Col2a1*, *Aggregan* and *SOX9*, increased deposition of collagen and GAG was frequently observed in alginate 3D bead cultures (518). Furthermore, enlarged matrix area was noted in E14.5 murine forelimbs cultured *ex vivo* at 5 kPa (242), indicating this effect is not confined to isolated cells.

Culture in 3D alginate beads presents a particular problem concerning O_2 diffusion and ultimately intracellular PO_2. Li et al. (341) addressed this question using a combination of experimentally determined O_2 consumption rates and PO_2 distribution modeling in scaffold-free aggregate cultures seeded at varying densities. They reported a 'critical' threshold of ~8 kPa, at which point cells no longer synthesized collagen and preferentially produced proteoglycans. How this relates to previous studies is unclear, given that the majority of the studies cited above use cells cultured in pellet or alginate 3D conditions at 5 kPa and report increased collagen deposition relative to room air cultures. Based on the PO_2 profiles published by Li et al., average pellet PO_2 may be closer to 8 kPa when cultured at room air (~18.5 kPa) than under 5 kPa, and therefore one might expect increased collagen deposition in these pellets. Despite this, gradients in collagen deposition have been observed in alginate beads under 5 kPa, with the highest collagen-associated staining apparent at the periphery (117). Thus, the concept of a critical threshold for collagen deposition may have some merit, but discrepancies in the actual PO_2 value are expected given differences in O_2 diffusion between scaffold free and alginate cultures.

5. Marrow-derived stem cells

The concept of a stem cell niche within the bone marrow and its influence on mesenchymal and hematopoietic stem cell fate are front-and-centre in our understanding of the physiology of these cells. Elegant imaging-based investigations have demonstrated that hematopoietic stem cells (HSC) preferentially localize to small arterioles within the endosteal marrow (319) and that variations from this niche induce a proliferative and 'hypoxic' phenotype (319, 430). Given our knowledge that the steepest O_2 gradients outside the lungs exist across the walls of arterioles (Section III-B and Figure 9), it is likely that steep gradients in PO_2 also exist in between arterioles within the bone marrow. Such heterogeneity in marrow PO_2 may be confounded by regional heterogeneity in cell populations, with varying degrees of cellular activity and thus O_2 consumption (552). Measurements of marrow PO_2 range from 1.7 to 7.2 kPa (Figure 16A), yet no study has directly addressed the concept of localized regions of lower PO_2. Moreover, mesenchymal stem cells (MSC) and hematopoietic stem cells (HSC) may originate from the bone marrow, but their sites of action extend throughout the body. Indeed, many have postulated that changes in PO_2 as cells exit the marrow into the systemic blood stream serve as a key trigger for terminal differentiation (402). In light of this, defining physiological normoxia for marrow-derived stem cells is difficult. Here, we will consider 3-5 kPa as normoxia but will discuss select studies in which cells were cultured under 2 kPa for comparative purposes. A full summary of papers meeting these criteria is provided in Table 1-9.

Expansion at a PO_2 of 3-5 kPa has repeatedly demonstrated greater colony formation or proliferation of mesenchymal stem cells (MSC) isolated from human (149, 528, 616) and sheep (700) bone marrow, although conflicting evidence has also been reported (449). A number of studies have identified an enhanced chondrogenic differentiation potential in MSC colonies expanded at 5 kPa (336, 374, 391, 528, 700), as characterised by the expression of *Col2a1* (see above) and quantitative analysis of glycosaminoglycan deposition. One study of human MSC cultured at 5 kPa reported no change in chondrogenic or osteogenic differentiation (449). This could be related to the source of cells used, as the latter study sourced human MSC from a commercial supplier whereas the majority of studies obtain cells directly from donors. A common observation reported by Pattappa et al. and others (149, 700) is a reduced level of cellular senescence after long-term expansion at 5 kPa. This finding may be related to the increased level of 'stemness' or differentiation potential in these cells, and supports the notion of a more physiologically relevant phenotype under physiological PO_2 *in vitro*. Fewer studies have focused on hematopoietic (CD44+) stem cells (HSC) within the marrow. Reykdal et al. observed less nitrite accumulation in cultures of human CD34+ HSC isolated and expanded at 5 kPa for 7 days. These authors attributed this to reduced induction of the inducible isoform of nitric oxide synthase, although they did not investigate the contributions of other isoforms. Similar to MSC, HSC proliferate more rapidly under 5 kPa and form larger colonies (483).

I. Skin

The measurement of transcutaneous PO_2 using non-invasive techniques has long been used experimentally and clinically as a readout of peripheral perfusion/oxygenation. This procedure involves placing a housed electrode onto the skin surface heated to 41-44^oC to encourage microvascular vasodilation, thus increasing local blood supply which creates a local excess of O_2 delivered by the arteriolar circulation (Figure 17A). Transcutaneous PO_2 is directly proportional to cutaneous blood flow (647) and inversely proportional to epidermal thickness (144). Heating the skin from 37-45^oC increases blood flow 3-4-fold (269), thus temperature intimately influences the reported PO_2 value. For example, transcutaneous PO_2 in the human forearm was recorded as 9.3 kPa at 44^oC by Falstie-Jensen et al., yet only 5.1 kPa at 41^oC by Spence et al. (550). Whilst a useful readout of arterial oxygenation clinically, such artefactual conditions result in relatively poor correlation with *bone fide* measurements of skin PO_2 (see Figure 17B). Using penetrating electrodes, PO_2 has been determined in the human abdominal, forearm, thigh and fingernail cuticular dermis, as well as in rat abdomen. At a generalised subcutaneous level, PO_2 has been reported to range between 3 (266), 4.1 (626), 5 (551), 5.3 (632), 6.3 (637) and 8 kPa (138). More detailed investigations by Evans and Naylor (140), Baumgärtl et al. (27) and Wang *et al.* recorded PO_2 profiles at varying dermal depths (see Figure 17C). Evans and Naylor reported PO_2 values of 6.1, 6.2 and 7.4 kPa at depths of 1-2, 3 and 5 mm respectively, and similar profiles are also reported at equivalent depths in other areas of skin (27, 632). Clearly, the skin has two distinct sources of O_2 diffusion, the atmosphere and microvasculature, and a nadir exists in which cells receive little O_2 from either. One apparent pitfall in skin PO_2 measurements using penetrating electrodes is the potential for overestimated values resulting from damage to the dense microvasculature. Jamieson and Brenk demonstrated that larger electrodes (330µm vs 60µm diameter) produced higher average subcutaneous PO_2 values in the rat abdomen (alongside similar measurements in liver, kidney, and splenic tissue), with evidence of macroscopic tissue damage.

More recently, significant efforts have been made to develop techniques to non-invasively monitor cutaneous PO_2 using phosphorescence quenching. Unlike the methods discussed in Section II-C, in which a synthetic porphyrin-type dye is administered exogenously as an O_2-sensitive probe, a technique developed by Mik and colleagues (223) employs 5-aminolevulinic acid (ALA), a precursor to the endogenously produced protoporphyrin IX. ALA cream is administered topically to the skin and results in accumulation of protoporphyrin IX in the mitochondria of epidermal cells within ~3 h (224). Using this technique, a PO_2 of between 6.3-8 kPa was reported in cutaneous mitochondria of the rat abdomen (223, 225, 637), which correlates well with similar measurements using microelectrodes (138, 139). By co-injection of the hydrophilic Oxyphor G4 dye, simultaneous measurement of mitochondrial and microvascular PO_2 was possible (223). A gradient of ~2.7 kPa

between blood and mitochondria was reported in the rat abdominal cutis, which was abolished by topical application of cyanide. Notably, a microvascular PO_2 of 10 kPa measured in this study is well within the range of reported transcutaneous PO_2 values (9.3-11.2 kPa (144, 560, 647)), confirming that transcutaneous measurements accurately reflect microvascular PO_2. In a more recent study, mitochondrial PO_2 was found to be a very sensitive indicator of the limit of hemodilution in pigs (489). However, the average baseline mitochondrial PO_2 value reported in this study (2.7 kPa) is far lower than that recorded in rats in previous investigations (7.3-8 kPa (223, 225)), a discrepancy that remains unexplained.

Fibroblasts isolated from mouse embryos, tail and human dermis have all been cultured at 3-5 kPa (Table 1-10). Common to many of these independent reports is the observation that cells maintained at low O_2 exhibit markedly less chromosomal instability/breakage (78, 283, 446) and senescence (78, 446). This was particularly apparent in mouse derived fibroblasts (446) which appear highly sensitive to the damaging effects of atmospheric conditions. Mouse embryonic fibroblasts (MEF) cultured at 3 kPa had higher and persistent expression of the p53-related proteins $p19^{ARF}$ and p16, yet senescence could be rapidly induced by re-exposure to 20 kPa (446). Notably, this sensitivity appears specific to laboratory rodent strains and is not observed in wild animals of the same species (448). This could reflect the longer telomeres observed in laboratory species (232) that are thought to occur in the absence of evolutionary selection for short telomeres in the wild. In human keratinocytes, isolation and culture at 4 kPa O_2 results in enhanced migration rates associated with reduced superoxide generation compared to cells cultured under 20 kPa (493).

Carrera et al. (68) reported increased HIF-1α activity in primary keratinocyte populations cultured at a PO_2 of 5 kPa for up to 4 days. This was reduced by inhibition of the MAPK cascade and with certain DNA damaging compounds such doxorubicin, with minimal effects on proliferation. It is likely that these observations can be attributed to the phenomenon of ‘self-inflicted hypoxia’ (see Section IV-D), based on the following observations: (i) HIF-1α protein levels were still minimal at 5 kPa compared to those observed at 1 kPa. Since monolayer oxygenation decreases with cell density (see Section IV-C), HIF-1α protein may only be stabilized at confluency and may therefore only affect proliferation in its latter stages; (ii) prominent HIF-1α protein expression and HRE-luciferase was observed in monolayers of the colon cancerous cell line HCT116 under the same conditions. Oxygen consumption rates are reportedly higher in these cells compared to primary human keratinocytes (25 vs 8 amol/min/cell (147, 619, 638)), and thus would have a lower intracellular PO_2 and hence higher HIF-1α activity; and finally (iii) inhibition of HIF-1α stabilization was observed when cellular activity was reduced by MAPK inhibitors or by DNA damaging agents. A reduction in cellular activity may result in decreased O_2 consumption rates, thereby increasing intracellular PO_2. As discussed in Section IV-D, an understanding of O_2 dynamics in monolayer

cultures is critical in interpreting experimental data, and should be considered when designing a model of *in vitro* normoxia.

J. Adipose tissue

Interest in PO_2 distribution in adipose tissue has increased in the last 2 decades (246) based on the observations that tissue from obese patients or animals can be hypoxic, although this is not fully accepted (188). In lean mice, PO_2 values have been reported between 6.4-7.7 kPa in epididymal fat pads (673, 676) and 5.6 kPa in the perigonadal white adipose tissue (477). Parallel measurements in the *ob/ob* transgenic obese mouse revealed a consistently lower adipose tissue PO_2 (2-4.6 kPa, decreasing with age), with strong histochemical evidence for tissue hypoxia through pimonidazole staining (477, 673, 676). In humans, subcutaneous adipose tissue PO_2 was measured at 7.6 kPa in the arm (280) and 7.3-7.9 kPa in in abdomen (187, 447) of lean patients. In contrast to rodent models of obesity, adipose tissue PO_2 in obese patients has been shown to be both higher (187, 618) and lower (280, 447) compared to matched lean patients. Pasarica and colleagues (447) were able to demonstrate that adipose tissue PO_2 correlated negatively with percentage fat composition and the expression of the inflammatory marker CD68, whilst correlating positively with capillary density. Based on these correlative relationships and the known histological characteristics of adipose tissue from obese patients (582), a lower PO_2 in tissue from obese patients seems logical. The work by Goossens and colleagues (39, 187, 618) reports a higher fasting adipose tissue PO_2 in obese patients compared with matched lean (~9 vs ~6 kPa) subjects (187) and that weight loss in obese patients is associated with a significant reduction in mean adipose PO_2 (6.8 to 5.5 kPa) (618). It was demonstrated that increasing and decreasing adipose tissue blood flow (using isoprenaline and angiotensin II infusion, respectively) leads to corresponding changes in tissue PO_2, corroborating claims by Pasarica et al. (447) concerning the relationship between adipose tissue PO_2 and perfusion/vascularization. As adipose tissue blood flow is reduced in obese patients (187), one would expect a lower PO_2. These discrepancies could be due to either differences in technique used to monitor PO_2, with Goossens and colleagues using a custom-designed microdialysis technique as opposed to the more commonly used electrode/fiberoptic probe, or physiological state in which measurements were made (fasting (187) vs normal (280, 447)).

As discussed and reviewed in by Trayhurn (586), a large body of work exists examining the effects of hypoxia (PO_2 of 1-2 kPa) on adipocyte function *in vitro* to better understand the pathophysiology of obesity. However, as highlighted by Trayhurn, culture at 20 kPa followed by a dramatic drop to 1-2 kPa has limited physiological significance, as neither the starting PO_2 nor 'hypoxic' value are physiological for adipocytes. It is far more likely that adipocytes experience a PO_2 of around 6-7 kPa *in vivo*, and to date two studies have attempted to address this issue. The first by Wood and colleagues (663) exposed primary human adipocytes to PO_2 values of 1, 3, 5, 10, 15 and 20 kPa

for 24 h and measured expression and release of the key adipokines leptin and adiponectin, as well as IL-6 and VEGF. A strong negative correlation was observed been leptin, VEGF and IL-6 mRNA expression and PO_2, whereas the opposite was observed for adiponectin and glucose uptake (663). Notably, the steepest part of the O_2 concentration response curve was between 3-10 kPa, the (patho)physiological range of PO_2 in adipose tissue (see above). However, adipocytes were only exposed to different PO_2 environments for 24 h, which may induce a response more representative of a response to low O_2 and less indicative of their physiology *in vivo*. This problem was addressed in a subsequent study, in which Famulla and colleagues (145) cultured adipocytes at 5 and 10 kPa for 14 days before measuring key adipocyte functions. Corroborating observations by Wood et al. (663), leptin expression and secretion were decreased at lower O_2 and associated with increased triglyceride content and glycerol release. Interestingly, peak responses were observed at a PO_2 of 10 kPa (145), conditions in which intracellular PO_2 is likely to be ~7 kPa based on known O_2 consumption rates of cultured adipocytes (694).

In addition to adipocytes and vascular cells, adipose tissue also contains a population of multipotent stem cells known as stromal cells (177). A number of studies have investigated the effects of long-term culture of these adipose-derived multipotent stem cells at low O_2 levels, as summarised in Table 1-11. Similar to other types of stem cells (see Sections H-5 and K-3), culture at either 2 (606) or 5 (464, 512, 533) kPa for the duration of culture/differentiation resulted in increased adipogenic (512, 606) and/or osteogenic/chondrogenic (464, 606) differentiation *in vitro*. Notably, enhanced differentiation potential *in vitro* did not translate into increased chondrogenesis when explanted *in vivo* (464). Whilst informative, the rationale behind culturing adipose derived stem cells at 2-5 kPa is unclear in light of the known distribution of PO_2 in such tissue *in vivo* (mean tissue PO_2 ~7 kPa). It is possible that populations of stem cells exist in localized regions of lower PO_2 within the tissue, although no evidence for this has been put forward to date. It seems more likely that 2 or 5 kPa O_2 was chosen as a 'best-guess' based on similar publications in cells of different origin. Although the matter may appear trivial, the work of Wood et al. (663) discussed above illustrates a steep relationship between ambient PO_2 and adipocyte function between 3-10 kPa, and thus selecting the most appropriate ambient PO_2 at which to culture such cells is absolutely critical.

K. Uterus and embryo

1. Uterus and oviduct

The uterus is a highly dynamic organ that experiences gross changes in physiology and function throughout the menstrual cycle, with surges in hormone secretion closely linked to contractile function and blood flow. In seminal work during the late 1960's, Mitchell and Yochim described periodic fluctuations in luminal (intrauterine) PO_2 that were rhythmic from hour to hour (401), and also changed dramatically according to stage of the menstrual cycle (400). During estrous,

intrauterine PO_2 has been reported at between 3 (677) and 3.4 kPa (288, 400, 401) in the rat, 2.7 kPa in the guinea pig (174), 4.9 kPa in hamster (155), 5.7 kPa in rabbit, 1.7 kPa in Rhesus monkeys (155) and between 2 (674) and 2.4-2.5 kPa in humans (440, 488). In experimental rodent models, intrauterine PO_2 peaked during the diestrous-1 period (288) and was strongly associated with similar increases in blood flow (174, 400). Such changes were not observed in primates (155). Upon conception, intrauterine PO_2 rises dramatically in rats (677) and rabbits (155) simultaneous with increases in progestin and decreases in estrogen secretion (677). Indeed, ovariectomy to mimic estrogen depletion also caused large increases in intrauterine PO_2 in rats (from 3.4 to 8.2 kPa) which were reversed by concomitant estrogen treatment (401). Unfortunately, no data exist to our knowledge concerning intrauterine PO_2 across the menstrual cycle in humans, nor are there direct measurements prior to and post conception. Endometrial PO_2 was measured by Rodesch and colleagues (488) in patients undergoing elective termination procedures at 8-10 weeks and in those at 12-13 weeks. These authors report an increase from a mean PO_2 of 5.3 kPa in the early termination to 6.2 kPa at 12-13 weeks. Similarly, Jauniaux and colleagues (271) measured PO_2 in coelomic/amniotic fluid and in the decidual lining in pregnancies between 7-10 weeks and 11-16 weeks. Whilst intrauterine fluid PO_2 was similar (2.5 kPa, similar to that reported by Ottosen et al. (440) and Yedwab et al. (674)), PO_2 in the decidual lining was significantly higher in late pregnancies (8.6 vs 9.8 kPa) (271). Thus, intrauterine PO_2 is relatively low prior to pregnancy and during estrous, especially in primates and humans, but rises rapidly within the uterine wall (myometrium/decidua) upon conception or during diestrous in line with increases in blood flow.

In the fallopian tubes, a dichotomy exists between mammalian species according to whether ovulation is cyclic or initiated by copulation. In rodent species (non-cycling), PO_2 within the fallopian tubes/oviducts remains constant and is relatively high, ranging from 6.8-8 kPa in rabbits (155, 380) and 5.6 kPa in hamsters (155). In contrast, PO_2 in the oviduct of rhesus monkeys is incredibly low when ovulation is not occurring (<1 kPa O_2) but increases dramatically during ovulation to between 7-10 kPa (155, 365). During passage to the uterus, the oocyte receives nutrients and O_2 directly from the fallopian tube lumen, and thus it is postulated that rodent oviduct PO_2 is maintained at a high level to ensure it can accommodate an oocyte at any point (365).

2. In vitro fertilization

One of the earliest adoptions of in vitro culture under physiological normoxia was in the field of in vitro fertilization, where the first successful culture of a human embryo to the blastocyst stage was achieved only under 5 kPa *(557)*. Since then, working under a reduced ambient PO_2 (usually 5 kPa) has become reasonably common both in research and clinical IVF *(45, 186, 285, 314, 418)*. Given the demand for improved clinical outcomes with IVF procedures, a large number of clinical trials have been performed to investigate whether culturing early-stage embryos at 5 kPa is

beneficial. These have been reviewed extensively in several meta-analyses *(45, 186, 418)*, and as such this topic will not be discussed in detail. In two reviews published in 2011, *(45, 186)* conflicting conclusions were drawn. Bontekoe and colleagues (45) reported a statistically significant improvement in live birth rate when embryos were cultured at 5-6 kPa O_2 which equated to an increase in success rate from 30% to between 32-43%. In contrast, Gomes-Sobrinho and colleagues *(186)* were unable to conclude that culture at low O_2 improved outcomes, although they did state that many results seemed promising. Notably, 7 studies were retrieved in both reviews based on their respective selection criteria, yet these were not the same 7 studies. Differences in selection criteria, with those applied by Bontekoe et al. (45) appearing stricter, are likely to explain the discrepancy in outcomes. In a later review by Nastri and colleagues *(418)*, a small (5%) but significant improvement was detected in live birth rates when embryos were cultured at 5-6 kPa, corroborating the findings of Bontekoe et al. (45). Common to all reviews was the criticism of study quality; especially related to investigator bias and differences in equipment/technique.

More recently, some have challenged the dogma that 5 kPa represents the ideal physiological PO_2 at which IVF should be conducted (286, 406, 670). This is based on the observation that PO_2 drops substantially between the oviduct and uterine lining; from ~6 to 2-3 kPa (see Figure 18), and thus the early blastocyst may experience a decrease in ambient PO_2 during implantation. To model this *in vitro*, embryos have been cultured at room air (670) or 5 kPa (286) until day 3, at which point ambient PO_2 was reduced to 2 kPa and blastocyst yield and quantity assessed at day 5. Cell yield increased 2-fold when embryos were cultured at 2 kPa between days 3 and 5 only when they were previously cultured at 5 kPa (286), with no significant effect detected if the embryo was previously kept at room air (670). In light of the significant effort and expense invested in improving IVF outcomes by utilising the concept of physiological normoxia, these more intricate efforts to better match physiological conditions are a logical progression.

3. Embryonic stem cells

Embryonic stem cells (ESC) are pluripotent stem cells derived from the inner cell mass of the early blastocyst, pre-implantation into the uterine wall, and receives nutrients and O_2 from the fallopian tube/uterine fluid. As such, they propagate in an environment containing low O_2, ranging between 2-5 kPa depending on the species (see above). Much like in IVF procedures, the derivation of ESC from the early blastocyst has long been conducted under low O_2 conditions (see (397) and (539) for reviews, and Table 1-12 for an up-to-date summary). This has largely been driven by enhanced proliferation or colony/embryonic body formation, consistently observed in both mouse and human ESC expanded at a PO_2 of 2-5 kPa (30, 142, 161, 173, 176, 300, 403, 467, 469, 473, 475, 629). A second advantage is a higher degree of pluripotency and less spontaneous differentiation; whether determined by expression of the OCT4/NANOG/SOX2 triad or downstream targets (643, 678), by SSEA-1 expression (142, 469), or morphological analysis (161, 469). Interestingly, although

maintained in an undifferentiated state, ESC at low O_2 can also more readily differentiate and to a higher degree when stimulated. More efficient differentiation into cardiomyocyte (30), vascular (320, 473) pulmonary (173), chondrocyte (307) and most often neuronal (300, 403, 404, 666) lineages has been reported. Differentiation into neural precursor cells appears to occur preferentially at very low PO_2 (2-3.5 kPa) under the control of HIF-1/2 (666).

Genomic stability has often been assessed in ESC cultured at low O_2 and compared with those exposed to room air. Forsyth et al. (161) showed that hESC exhibited greater spontaneous chromosomal aberrations after 20 passages at room air compared to those maintained for the equivalent time at 2 kPa, which had very few additional aberrations. Lengner et al. (335) subsequently demonstrated that culture at room air is responsible for the premature and irreversible inactivation of an X-chromosome frequently observed during hESC culture, through demethylation of the *XIST* gene promoter region. Similarly, hyper-methylation of the DLK1-DIO3 cluster at room air results in silencing of this gene region which can be prevented by maintenance at physiological PO_2 levels (5 kPa).

Embryonic stem cells are known to be highly metabolically active, preferring glycolysis as their source of ATP generation (79). This phenotype appears accentuated when ESC are cultured at low O_2, with higher rates of glucose consumption and lactate production frequency observed (83, 158, 596). In their most recent paper, Christensen and colleagues (83) propose that increased glycolysis is facilitated by enhanced expression of glucose transporter 3 (GLUT3) which correlated positively with OCT4 mRNA expression, thus relating glycolytic activity to pluripotency. ESC are also known to consume a large amount of O_2, which creates a concern regarding self-inflicted hypoxia during culture (see Section IV). It has been demonstrated on a number of occasions that O_2 can be severely limited in monolayer cultures of ESC especially at low ambient PO_2 (320, 466, 467, 596). Hence, as proposed by Turner et al. (596), confluent cultures kept at a PO_2 of 2 kPa (and even perhaps at 5 kPa (2, 320)) are inevitably encroaching on anoxia. This issue, whilst acknowledged, is yet to be satisfactorily addressed and thus calls into question the physiological validity of using very low (2-5 kPa) ambient PO_2 in ESC culture, with an intracellular PO_2 ~2-5 kPa being the actual desired outcome. Limit studies have been conducted with ESC cultured at higher (6-10 kPa) O_2 levels, which may counteract O_2 consumption to produce a more physiological intracellular PO_2. In mouse ESC, similar levels of proliferation and pluripotency (OCT4/NANOG/SOX2 mRNA) (467) and VEGF production (473) were observed between ~4-10 kPa ambient PO_2. However, human ESC differentiated to a higher degree during colony growth at 10 vs 5 kPa ambient PO_2 (469). Thus there may well be physiological consequences to the 'self-inflicted' hypoxia occurring following culture at 2-5 kPa, although this needs further investigation.

4. Placental tissue and trophoblasts

Placental development and subsequently the health of the tissue and fetus are closely linked to changes in PO_2 (270, 597, 650). During embryogenesis, rapid proliferation of trophoblastic tissue creates thick villus membranes which result in reduced diffusion of O_2 from the maternal arterial blood to the surrounding tissue. As such intervillous PO_2 is reportedly as low as 2-3 kPa in human placenta at 8-10 weeks gestation (271, 272, 488), which has been shown to modulate the proliferative and angiogenic trophoblastic phenotype required to establish a patent intervillous circulation (65, 270, 597). Once adequate perfusion is established (>12 weeks gestation), a rapid rise in villous/placental tissue PO_2 is observed, reaching 7-8 kPa (271, 272, 488, 548), where placental tissue PO_2 remains largely stable for the duration of gestation (597).

The issue of oxygenation *in vitro* has long been considered important in the culture of trophoblasts and placental tissue explants, and a large amount of the literature has already been elegantly reviewed by Tuuli and colleagues (597). Numerous studies have attempted to understand the impact of changes in PO_2 on trophoblast phenotype, largely through comparative studies on cultures maintained at ambient PO_2 values ranging from 20 to <1 kPa. However, measurement of pericellular PO_2 in primary human trophoblast cultures using a microelectrode revealed that cells were experiencing near anoxic (PO_2 of 0.1-0.6 kPa) conditions even when cultured under ambient (20 kPa) PO_2 (77). This gradient was medium-depth dependent and abolished by methanol fixation of the cells, confirming evidence discussed previously (Section IV-C). Although not confirmed by others to date, these data clearly call into question the validity of using standard culture conditions to study primary trophoblasts *in vitro*.

VII. Conclusions and future prospects

The concept that oxygen is critical for normal cellular function is unanimously accepted. However, for scientists O_2 may represent more of a double-edged sword, in view of the numerous damaging effects associated with reactive species produced from O_2. Despite this, traditional methods used extensively in the culture of mammalian cells result in the exposure to undefined, uncontrolled O_2 levels which are often at either extreme of the quasi-physiological range, i.e. either hyperoxic or hypoxic with respect to the physiological milieu. In this review, we have provided an up-to-date assessment of the current understanding of PO_2 distribution in tissues and organs *in vivo*, and discussed some examples of where this knowledge has been implemented to enhance the physiological relevance of cell culture conditions *in vitro*. In collating and critically discussing this information, we hope to provide a reference source for future research aimed at designing novel and physiologically relevant experiments to address ever-more complex scientific questions.

As highlighted throughout this review, accurately recapitulating physiological normoxia *in vitro* is far more complex than simply adjusting the ambient 'head-space' PO_2. An understanding of the physical conditions in which cells are cultured (i.e. plastic permeability, volume of medium used

and rates of O_2 equilibration in solution) should underpin the choice of the final desired pericellular PO_2 determined using mathematical models and/or experimental data to predict pericellular versus ambient PO_2. Such careful experimental design informed by our review of the literature should provide researchers with the 'closest representation' of likely tissue PO_2 levels *in vivo*. Differences in experimental conditions and protocols for cell culture, when sufficient detail is reported, and concerns about the definition of physiological normoxia have may limit collective advances for translational research.

Powerful tools are now available to better predict and measure PO_2 in culture, with accumulating studies highlighting their necessity. We thus hope that the contents of this review will help unify experimental approaches used by basic and clinical researchers. In this context, we propose the following guidelines for researchers who wish to culture their cells under defined physiological normoxia *in vitro*:

1. **Define the conditions appropriately.** Oxygen levels should be measured and defined in terms of PO_2 whenever possible. Often reported as 'room air' or 20% O_2, most cell culture experiments are conducted in incubators in which the ambient PO_2 is closer to 18.5 kPa, and sometimes below.
2. **Start the experiment properly.** Always pre-equilibrate all culture media and solutions for a minimum of 2 hours, and considerably longer for large volumes, at the appropriate ambient PO_2 prior to use on cells. It is advisable to store all culture plasticware at the desired PO_2 to avoid O_2 leaching.
3. **Controlling monolayer oxygenation.** We have highlighted throughout this review that appropriately controlling monolayer oxygenation is a consistent problem. There are several parameters that can be easily altered to maintain control over monolayer oxygenation, listed here according to relative ease:
    i. Use of O_2-permeable plastic: this creates a new and much more efficient way in which O_2 can be delivered to the monolayer, alleviating problems due to static medium.
    ii. Varying medium height: surface area: by minimising the ratio between medium height and culture surface area, one reduces the distance for O_2 diffusion and thereby maximise delivery. Simply reducing the volume of medium can achieve this.
    iii. Varying ambient PO_2: if medium volume cannot be reduced, one can increase the ambient PO_2 to compensate for monolayer O_2 consumption.
    iv. Continuous perfusion: To completely alleviate issues associated with O_2 delivery, continuous perfusion of well oxygenated medium is an elegant way to control intracellular O_2 precisely even in response to large increases in O_2 consumption.

    Results should always be interpreted in the context of pericellular or intracellular PO_2 rather than ambient PO_2. Mathematical models have proved a useful tool to predict such values,

and recent advances in experimental techniques have allowed the direct monitoring of intracellular PO_2.

4. **Consider a range of PO_2 values.** Oxygenation *in vivo* is heterogeneous in both space and time, and thus no single value can be described as normoxia for a given cell type. Moreover, the expression and activity of many proteins are not linearly related to the intracellular PO_2. It is for these reasons that we find it far more informative to conduct preliminary studies under a range of 'physiological' O_2 levels (2-10 kPa, for example), which offer a far more relevant comparison than 21 vs 1 kPa.

Can culture under physiological normoxia improve the translation of cell physiology to animal models and the clinic? There are several excellent studies (342, 499, 553, 578) that have already provided convincing evidence in favor of this hypothesis, and the concept is already implemented clinically in IVF clinics around the world. Despite this, we believe there is still significant scope to demonstrate that culturing cells under their respective physiologically normoxic conditions genuinely recapitulates their native phenotype and improves outcomes of clinical relevance. Moving forward, we believe it is critical for this field of research to avoid isolated phenotypic comparisons between cells at room air and those at normoxia, instead focusing on whether culture under physiological conditions can reveal novel insights into disease pathology, or pharmacodynamics and kinetics of existing and novel therapeutics. Moreover, it is perhaps overly simplistic to consider only one normoxic value within a given tissue, when there is clearly considerably complexity in tissue oxygenation. Thus, the oxygen-sensitivity of a pathway of interest may be more accurately assessed over a small range of normoxic values, such as that between the renal medulla and cortex (Section VI-F) or from the inner bone marrow to the associated vasculature (Section VI-H). Furthermore, whilst critically important, O_2 represents just one component of the physiological milieu that is altered during standard culture practice, with other factors such as biomechanics, 3D co-culture and substrate matrix contributing equally to the cellular phenotype. Moving forward, it is essential to better model all these factors together to truly begin recapitulating the physiological environment *in vitro*.

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Figure Legends

Figure 1. Timeline of O_2-related research publications

(*Main* graph) Number of PubMed entries in which the keyword oxygen is mentioned within the title or abstract over the past 76 years. Arrows indicate important events relevant to the scientific study of oxygen in the context of medical science. (*Pie chart*) Distribution of PubMed entries in which 'oxygen' is mentioned in either the title or abstract between 01/01/2010 and 31/12/2017. ROS – reactive oxygen species

Figure 2. Techniques to determine tissue and cellular PO_2

(A) Diagram of the two common types of O2-sensitive electrodes, the Clark and Whalen type. Both are connected to an external power supply and galvanometer to record changes in current due to reduction of O_2 at the cathode. (**B**) The basis for hemoglobin saturation measurements. The binding of O_2 to hemoglobin is associated with a change in its spectral properties, which are measured at various wavelengths to determine the ratio of oxyhemoglobin to total hemoglobin. (**C**) *Top*, the excitation (solid)/emission spectra (dashed) spectra for Pt^{II}-porphryin based phosphorescent probes, with a structural schematic provided in the inset. Adapted from (151). *Bottom,* schematics illustrating the basis for O_2 measurements by time-resolved fluorescence; *left*, After the initial excitation flash, phosphorescent emission decays at an exponential rate, with most O_2-sensitive complexes exhibiting lifetimes in the 10-1000μs range; *right*, the relationship between calculated probe lifetime and PO_2. (**D**) Representative images of ^{11}C-acetate and ^{64}Cu-ATSM loaded isolated hearts subjected to left-anterior descending artery occlusion, generating a region of ischaemia identified by the absence of flow (^{11}C +ve) and +ve ^{64}Cu-ATSM loading. Taken with permission from (169). (**E**) Immunohistochemical section of a squamous cell carcinoma stained to show pimonadazole (green) and carbonic anhydrase 9 (red), with blood vessels (white) and necrotic regions (enclosed in dashed white) identified. Taken with permission from (253).

Figure 3. Oxygen gradients in the respiratory system

Concentrations, in kPa, of O_2 (●, left axis), CO_2 (■, right axis) and H_2O (▲, right axis) from 'dry' atmosphere to the pulmonary venous circulation. Increases in PH_2O and PCO_2 in the respiratory tract reduce PO_2 proportionally, and venous admixture within the pulmonary vein reduces PO_2 further.

Figure 4. Oxygen gradients in the vascular system

(**A**) The relationship between PO_2 (●, *right axis*), area (●) and velocity (●) throughout the vasculature. Highlighted are the generally perceived vessels in which gaseous (red) and nutrient (blue) exchange takes place. Velocity and area are arbitrarily defined here, and average PO_2 is adapted from Tsai et al. (2003). (**B**) A standard oxyhemoglobin saturation curve, with the average PO_2 values within relevant blood vessels annotated.

Figure 5. Parameters of atmospheric O_2 levels within the cell culture environment

(**A**) An approximate O_2 unit conversion table with the multiplication factor required to convert current unit (row) into desired unit (column). (**B**) The atmospheric composition at sea level, at 1600m above sea level, and within a standard culture incubator (5% CO_2, 75% humidity). (**C**) The P_{H2O} and PO_2 in pure water are plotted across a range of temperatures, and the concentration (cO_2) and solubility (S) of O_2 calculated based on the equations of Benson and Krause (32), as summarised by Forstner and Gnaiger (159). (**D**) An application of Fick's second law of diffusion to describe the time required for culture medium to equilibrate following exposure to an atmosphere of different O_2 content. Q=rate of diffusion, A=liquid surface area, D=diffusivity of O_2 in liquid, k=solubility of O_2 in liquid, Cs=saturating concentration, C=new concentration, d=liquid depth.

Figure 6. Predicting pericellular PO_2 in cell monolayers

(**A**) Parameters critical in predicting pericellular PO_2 in cultured cells. The value of V (O_2 consumption rate) can be simplified by considering a zero-order relationship between V and PO_2, as described by Yarmush et al. (672) or modelled using Michaelis-Menton kinetics as described by Powers et al. (466). As we know O_2 consumption is affected by O_2 availability, predictions will have more accuracy if V is modelled using the latter. (**B**) Illustration of the relationship between pericellular PO_2 and cellular O_2 consumption, with commonly used cell types annotated according to published values. Cell types with high O_2 consumption such as cardiomyocytes or hepatocytes can initiate a 'self-inflicted' hypoxia when ambient PO_2 is low. *Abbreviations:* P=pericellular PO_2; P_s=PO_2 at gas/liquid interface; d=fluid height; V=O_2 consumption rate (V_{max}); p=cell density; D=O_2 diffusivity in liquid; k=O_2 solubility in liquid; A=surface area at gas/liquid interface

Figure 7. Oxygen distribution in the lung

(**A**) An illustration depicting the known distribution of PO_2 in the mammalian lung, combining experimentally derived values with theoretical values derived from established physiology. Pulmonary arterial PO_2 (PaO_2) is usually slightly lower than alveoli PO_2 as outlined in the equation PaO_2 = PO_2(alveoli) – 1 kPa and discussed in Section III-A. (**B**) Whole lung PO_2 mapping using ^{3}He-based MRI scanning in healthy volunteers and in patients with chronic obstructive pulmonary disease (COPD). Taken with permission from Miller et al. (2010) (396). (**C**) Peribronchial PO_2 values determined using microelectrode penetration upon (*left*) ligation of the bronchial artery (BA) or bronchial collaterals in dog (adapted with permission from (282)), and (*right*) increases in forced inspiratory PO_2 (FiO_2), adapted from Herold et al. (1998) (236). Both examples illustrate the dependence of peribronchial PO_2 on bronchial artery perfusion rather than the bronchi themselves. (**D**) Comparison between conventional cell culture, in which the monolayer is completely submerged in medium, and air-liquid interface culture used to maintain differentiated primary

epithelial cells. Transwell® chambers are used to elevate the monolayer, exposing the apical surface to the atmosphere whilst maintaining basolateral access to medium through a microporous membrane.

Figure 8. Oxygen distribution in the arterial wall

(**A**) Illustration of known distribution of PO_2 in the wall of large arteries such as the aorta, carotid and femoral arteries, combining experimentally derived values with theoretical values obtained from established physiology. (**B**) Representative trace obtained when a microelectrode is advanced in 5µm increments through the wall of the rabbit thoracic aorta. Redrawn with permission from Niinikoski et al. (1973). (**C**) Graphical PO_2 profiles through the walls of small (*top*) and large (*bottom*) arteries, illustrating the effect that the vasa vasorum has on adventitial PO_2. *endothelium

Figure 9. Oxygen distribution in the microvasculature

(**A**) An idealised microvascular network. (**B**) Reported PO_2 values plotted against the vessel diameter in microvascular experimental preparations. Results are separated according to tissue, and coded according to the measurement technique employed: □ indicate microelectrode penetration, ∆ derived from measurements of hemoglobin saturation, where PO_2 was interpolated based on known species Hb dissociation curves, and ◯ phosphorescence quenching microscopy. Adapted from (594).

Figure 10. Effects of physiological normoxia on human endothelial cells

Experimental data describing the careful definition of cellular oxygenation *in vitro* and the subsequent characterization of cellular function and phenotype during long-term culture under these conditions. (**A**) Intracellular PO_2 in HUVEC monolayers cultured in 96-well plates (100µL volume) at a PO_2 of 18 kPa and then exposed acutely to 5 kPa, measured using MitoXpress INTRA in an O_2-regulated plate reader. This illustrates that the PO_2 in the medium is only moderately higher than cytosolic, and both parameters rapidly change upon changes in ambient PO_2. (**B**) Intracellular PO_2 in HUVEC monolayers as a function of the ambient PO_2, defined by a 4-parameter sigmoidal fit. Dashed lines indicate the conditions in which HUVEC were subsequently cultured. (**C**) HIF-1α protein levels in HUVEC cultured for 5 days at the indicated PO_2 in an O_2-regulated workstation, and corrected for the resulting intracellular PO_2 determined in **B**. Plotting against intracellular PO_2 results in P_{50} values more likely to be observed in the vasculature *in vivo*. (**D**) Expression of a Nrf2 regulated antioxidant defense protein, NAD(P)H-quinone oxidoreductase 1 (NQO1), in HUVEC cultured at a PO_2 of 18 or 5 kPa for 1-5 days and then challenged with the electrophile diethylmaleate (DEM, 100µM for 24h). These data illustrate the difference between cellular phenotypes when cells are exposed acutely (24h) or chronically (5 days) to lower O_2. (**E**) Phosphorylation of endothelial nitric oxide synthase (eNOS) in response to histamine (10µM, 2 min) stimulation in HUVEC cultured at a PO_2 of 18 or 5 kPa for 5 days, demonstrating a more rapid

decline in phosphorylation over time in cells cultured under physiological normoxia.. (**F**) Intracellular Ca^{2+} mobilization in response to histamine, measured using Fura-2AM. Lower plateau $[Ca^{2+}]_i$ at physiological normoxia are reversed by treatment with the sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor cyclopiazonic acid (CPA). Panels **A**, **B** and **D** are replotted from Chapple et al. 2016 (75) and panels **C**, **E** are taken from Keeley et al. 2017 (290), and panel **F** is taken from Keeley et al. 2017b (291).

Figure 11. Oxygen distribution in the adult heart

Illustration depicting the PO_2 within the heart, summarizing published studies for different species with known values established in physiology. Individual myocardial PO_2 values are further summarized in the adjacent graph, separated according to technique used; □ microelectrode penetration, △ derived from measurements of myohemoglobin saturation, where PO_2 was interpolated based on known species Hb dissociation curves, ○ phosphorescence quenching microscopy and ◊ implanted EPR probes. Values are colour-coded to highlight those taken at superficial sites (red) or single cell intracellular measurements (blue).

Figure 12. O_2 distribution in the brain

Illustration depicting the reported PO_2 distribution within the brain, with PO_2 values concerning microvascular or grey matter arranged according to reported depth of measurement. In some cases, depth was estimated based on the methodological description. Studies marked with a ○ were conducted using 2-photon PQM (define), whilst those marked with a □ used ESR.

Figure 13. O_2 distribution in the liver

Illustration of the reported PO_2 distribution in the liver. Note, blood entering the sinusoids is a 3:1 mixture of portal venous and hepatic arterial blood, thus sinusoidal O_2 concentrations can be estimated based on known values for the source vessels (PO_2 ~8 kPa).

Figure 14. O_2 distribution in the kidney

Anatomical illustration of known PO_2 values in the kidney, derived from a number of different experimental models, species and techniques. *Bottom left,* a representative trace obtained from invading an O_2-sensitive microelectrode into the rat kidney following the illustrated path. Depths corresponding to the cortex and medulla are highlighted. Adapted with permission from (362). *Bottom right*, PO_2 values within an individual rat nephron, adapted with permission from (642).

Figure 15. O_2 distribution in the gastrointestinal tract

A, Data from a number of studies (see text for more discussion and references) investigating PO_2 distribution in the gastrointestinal tract. Values are separated into those taken from the stomach, small and large intestines, and further divided according to anatomical/cellular location (lumen,

mucosa or serosa). Symbols are coded according to technique employed; □ microelectrode, △ approximate based on pimonidazole staining, ▽ suffusion, ○ phosphorescence quenching microscopy and ♢ EPR. **B,** Immunohistochemical images of the colon wall stained with the hypoxia-specific probe pimonidazole. Images are taken using either fluorescence (left) or bright field (right) microscopes. Taken with permission from (294) and (157), respectively.

Figure 16. Oxygen distribution in bone and cartilage

(A) Distribution of O_2 within a typical synovial joint. (**B**) Representative image highlighting the passage of a microvessel (yellow) from the outer bone (blue) into the marrow (black). Intravascular PO_2 was determined at 2 different points: within the bone (red arrow and dot) and within the marrow (green arrow and dot). IV – intravascular. Adapted with permission from (552).

Figure 17. Oxygen distribution in the skin

(**A**) Concept of transcutaneous measurement of blood gas concentrations. Heating the skin causes vasodilation and enhances O_2 delivery to the epidermis, where it diffuses towards the measurement electrodes. (**B**) Distribution of published PO_2 values in the dermis and subcutaneous tissues, as well as transcutaneous measurements. Symbols are coded according to technique used: □ microelectrode, ○ phosphorescence quenching microscopy. *Skin was heated to 41^{o}C in this study. # Paraffin oil was applied to the skin to prevent O_2 diffusion from the atmosphere, +Subcutaneous microvascular measurements using Oxyphor G4. (**C**) An example of a PO_2 profile generating by electrode penetration of the skin, adapted with permission from Baumgärtl et al. (27).

Figure 18. Oxygen distribution in the oviduct and uterus

Reported values for intrauterine and oviduct PO_2 across mammalian species. Intrauterine values are separated according to menstrual cycle stage. In Rhesus monkeys, ovulation is associated with large fluctuations in oviduct PO_2 (365), hence the pre-ovulation value is labeled appropriately.

Table 1-1 Permeability of commonly used plastic culture surfaces

Surface	D (10^{-5} cm^2 sec^{-1})	k (10^{-9} mol cm^{-3} $mmHg^{-1}$)	Reference
Medium	3.3	1.2	**(371, 389)**
	2	1.2	**(672)**
	2.8	1.3	**(112, 466)**
Polystyrene	0.01	8.6	**(466)**
PDMS	2.2	12.2	**(466)**
Teflon	0.03	5.1	**(311)**

Abbreviations: PDMS, polydimethylsiloxane; Teflon, polytetrafluoroethylene; D, O_2 diffusivity; k, O_2 solubility coefficient

Table 1-2 Equations used to predict pericellular PO_2 *in vitro*

Equation	Reference
$h = k\,[(P_S-P)/V]$	(559)
$-D\,dP/dx = VpP/[k(K_m+P)]$	(672)
Solved: $\theta = 0.5\,[1-\alpha-\beta_M + [(1+\alpha+\beta_M)^2 - 4\alpha]^{0.5}$	
$P=P_s - (Vp/60D)(760/k)(h/A)$	(389)
$-D\,dP/dx = -Dk(P_x-P_s)/h$	(371)
$1-\theta = [F_V+(1-F_V)(\theta/\beta_V+\theta)](\theta/\beta_M+\theta)\alpha^*$ (Solved)	(466)
$p= [(P-P_s)DA]/Vh$	(596)

Abbreviations: P= pericellular PO_2; P_s=PO_2 at gas/liquid interface; d=fluid height; V=O_2 consumption rate (V_{max}); p=cell density; D=O_2 diffusivity in liquid; k=O_2 solubility in liquid; K_m=PO_2 when O_2 consumption is 0.5max; A=surface area; $\theta = P/P_s$; $\alpha = dVp/DkP_s$; $\beta_M = K_m/P_s$; $F_V = V_{max,0}/V_{max,\infty}$; $\beta_V = K_V/P_S$ (where K_V is a fitted constant describing dV/dP_s)

Table 1-3 Pericellular PO_2 levels under standard culture conditions

Cell type	Method	PO_2 (kPa)		Reference
		Ambient*	Pericellular	
Rat hepatocytes	electrode	18 13 4	8 5.8 1.5	(250)
HepG2/3B	electrode	18	<0.1	(659)
HepG2/3B	electrode	18	<0.1 <0.1	(389)
BAEC	electrode	18	10.4	(389)
HUVEC hEPC	Fibre-optic probe	21 5 1	19-20 3.4 0.7	(2)
HASMC	ESR	21	18.9	(442)
HUVEC	MitoXpress	18.5 5	~17 3.5	(75)
hiPSC-CM HASMC HCF HMVEC Rat-CM	electrode	21	10.4 16.4 18.6 17.9 14.9	(521)
mESC	Fibre-optic probe	2	2	(404)
hESC iPSC	Fibre-optic probe	21 5 1	4-5 1.5 0.4	(2)
MEF	MitoXpress	21 10	8.1 3.8	(151)
hESC	Fibre-optic probe	21 5	15 ≤1	(320)
Rat mesangial LLC-PK_1	electrode	18	14.7 <0.1	(389)
Type II alveolar	electrode	18	13.3	(371)
Dermal fibroblasts	electrode	18 2	12 0	(581)
T-47D	electrode	21	1-18	(453)
MIN6 (β cells) PANC-1	Pimonidazole HIF-1α	20 10 7 5 3 0	- - + ++ ++ +++	(511)
Trophoblast	electrode	21	<1	(77)
hESC	Fibre-optic probe	21 5	15 ≤1	(320)
PMN	Oxodish	21 4	0-14 <1	(64)
RAW 264.7	Patch probe	20 5	~8 ~1.5	(690)
Caco-2	Patch probe	19 1	~4-5 <0.1	(114)
Cortical neurons	MitoXpress	18-20	14-16	(250)

*Unless clearly defined, ambient PO_2 was determined to be either 21 or 18 kPa based on the inclusion/absence of 5% CO_2 and humidification. *Abbreviations:* BAEC, bovine aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; HMVEC, human cardiac microvascular endothelial cells; HASMC, human aortic smooth muscle cells; hEPC, human endothelial progenitor cells; hiPSC-CM, human induced pluripotent stem cells-cardiomyocyte differentiated;; mESC, mouse embryonic stem cells ; hPSC, human pluripotent stem cells; HCF, human cardiac fibroblasts; Rat-CM, rat cardiomyocytes; PMN, peripheral blood mononuclear cells

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Species	Cell type	PO_2 (kPa)	Time (d)	Summary*	Reference
Human	HUVEC	5 (12 shear)	3	↑ growth (BrdU) ↑ expression of membrane and ECM proteins CD105, CD31, fibronectin, laminin and type VI collagen Different morphology after 24 h shear stress (20 dyne/cm^2) (more spindle-like, elongated) • ↑ NO and PGI_2 production with shear stress, ↔ with 20% vs 12% O_2	**(695)**
	EPC HUVEC	5, 1	3	dissolved medium O_2 level much lower in ESC, iPSC and EPC cultures than HUVEC • ↑ traditional hypoxia-sensitive genes (VEGF, Glut1, EPO)	(2)
	HUVEC HUAEC	3	up to 25	↑ FGF and VEGF stimulated growth and migration Putting non-adapted cells in 3% O_2 for 1 day ↑ effects of FGF and VEGF, and putting adapted cells in 21% O_2 did not reverse the effect of adaptation of growth. ↓ cell surface area compared to non-adapted once adhered • Micro-array analysis showed 41-74 up-regulated and 21-86 down-regulated genes (all HIF regulated), (HUVEC-HUAEC).	**(276)** **(277)**
	HUVEC	5 (5 shear)	3	↔ protein/mRNA expression following shear between 5 and 20% O_2. ↓ apoptosis gene expression (Bcl), no additive effect of 5-F**U** • ↑ permeability with 5-FU, reversed by resveratrol.	(1)
	HUVEC	5	1, >5	Culture at 5 kPa ambient PO_2 results in intracellular PO_2 of ~3.5 kPa in HUVEC monolayers ↓ Induction of antioxidant defense proteins (HO-1, NQO1) by electrophilic agents ↔ induction of glutathione-related proteins (GCLC, GCLM, xCT) but no change in $[GSH]_i$ ↑ Bach1 protein and siRNA-mediated knockdown rescued HO-1 induction at 5 kPa O_2 ↓ proliferation	(75)
	HUVEC	5, 1	>5	IC_{50} for HIF-1α stabilization is 2.3 kPa O_2 when plotted against intracellular PO_2 ↑ PP2A-C protein and co-localization with eNOS mediated rapid dephosphorylation in response to Ca^{2+} dependent stimuli, but not laminar shear stress. PP2A-C protein levels show bell-shaped relationship with ambient PO_2, peaking at 5 kPa NO bioavailability inversely proportional to PO_2 • Culture at physiological normoxia induced phenotype distinct from that under hyperoxic (room air) and hypoxic (1 kPa O_2) conditions.	**(290)**
	HUVEC	5	>5	↓plateau phase $[Ca^{2+}]_i$ in cells at 5 kPa O_2, rescued by co-treatment with SERCA-inhibitor CPA. • Cells at 5 kPa O_2 more resistant to Ca^{2+} overload due to mitochondrial 'sparing' by increased SERCA activity.	**(291)**
	HPMVEC ST1.6R	5	12	Confluent monolayers contained 30% fewer cells, but formed a tighter barrier as measured by TER. • ↑ cortical actin levels	**(547)**
	HDMVEC	5	14	↑ Basal and stimulated growth (PMA and IL-1β) • ↑ ECM proteins (type VI collagen)	**(697)**

Bovine	Corneal EC	6	4	• ↑ [H^3]-thymidine incorporation and cell count	**(684)**
	BRMVEC	95, 40, 20, 10 5	4	• Endothelial cells more sensitive to effects of oxygen compared with pericytes and fibroblasts from same vessel	**(490)**
Rat	Liver sinusoidal EC	5	5	↓ basal apoptosis (MTT and Annexin V/PI) ↔ morphology of confluent monolayer, or fenestrated characteristics ↓ endocytotic capacity during culture significantly less at 5% ↓ ICAM-1 and IL-6 expression/production (pro-inflammatory) and ↑ IL-10 production (anti-inflammatory) (only measured after 48 h) ↑ H_2O_2 basal production (DCF), treatment with catalase reverses effects on apoptosis • ↔ NO release (Griess reaction)	**(377)**

Abbreviations: HUVEC, human umbilical vein endothelial cells; ECM, extracellular matrix; NO, nitric oxide; PGI_2, prostacyclin; EPC, endothelial progenitor cells; iPSC, inducible pluripotent stem cells; ESC, embryonic stem cells; VEGF, vascular endothelial growth factor; Glut1, glucose transporter 1; EPO, erythropoietin; HUAEC, human umbilical artery endothelial cells; FGF, fibroblast growth factor; 5-FU, 5-fluorouracil; HO-1, heme-oxygenase 1; NQO1, NADPH-quinone oxidoreductase 1; GCLC/M, glutamate-cysteine ligase catalytic/modulatory subunit; xCT, cystine-glutamate antiporter system X; GSH, glutathione; PP2A-C, protein phosphatase 2A catalytic subunit; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; CPA, cyclopiazonic acid; HPMVEC, human pulmonary microvascular endothelial cells; TER, transendothelial resistance; HDMVEC, human dermal microvascular endothelial cells; PMA, phorbol 12-myristate 13-acetate; IL-1β, interleukin 1β; BRMVEC, bovine retinal microvascular endothelial cells; ICAM-1, intracellular adhesion molecule 1; DCF, dichlorofluoroscein; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; PI, propidium iodide.

4277 **Table 1-5 The effects of culture at physiological normoxia on circulating cell physiology**

Species	Cell Type	PO_2 (kPa)	Time (d)	Summary	Reference
Human	PBMC	5	1-5 d	• ↑unstimulated and Con A stimulated proliferation, no effect on PHA stimulation • ↑early expression of lymphocyte marker CD69 • ↓basal and H_2O_2 induced ROS production (DCF) • Altered released cytokine profile following stimulation with Con A or LPS	(315)
	PBMC	5	15	• Gene expression array revealed higher expression of genes important for lymphocyte biology, immune function and cell-cycle progression. • ↓expression of genes involved in stress response, cell death and cellular repair.	(210)
	PBMC	5	5 d	• Proliferation stimulated by antigen CD8/CD28 crosslinking, or Con A, was ↑at 5%, but no effect on phytohemagglutinin-stimulated proliferation. • Unstimulated cell viability unaffected by adaptation to 5%	(16)
	Negatively enriched T cells PBMC	5	3 d	• ↓GSH and GSH:GSSG following culture (vs fresh), less reduction at 5% • ↓proliferation • ↑CD8/CD28 stimulated NO and ROS production • ↑lymphocyte marker CD69	(17)
	PBMC	5	>6 d	• HIV-transactivator of transcription (Tat) protein induces apoptosis at 20%, but proliferation at 5% O_2 • Tat can be used to prime cells for HIV infection as well as other methods, only at 5% O_2	(499)
	PBMC	5	7 d	• *in vitro* culture ↑SOD1-3, CAT, GPx mRNA. At 5% O_2, ↓CAT and GPx mRNA vs room air • ↑ GPx activity, no change in CAT or SOD activity. • ↑ Intracellular GSH, though overall decrease during culture. • ↓superoxide and hydrogen peroxide	(146)
	PBMC-matured dendritic cells	5	duration of culture	• ↔ total cell yield • ↓endocytosis of dextran-FITC • ↑LPS-induced IL-12 (p70) and T-cell activation during co-culture	(171)
Mouse	CD4+ T cells	5, 1	36 h	• ↑Th17 specific differentiation at 5%, ↓ all subset differentiation at 1% • No effect of 5% on proliferation, 1% ↓ • Increased mTORC1 activity at 5% O_2	(259)
	Primary splenocytes Splenocyte-differentiated T Cells	2.5	6 d	• ↑CD4:CD8, but dramatically higher killing (lytic) potential of CD8+ cells at 5%, with ↑density of CD25 surface antigen. • ↓cell death	(61)
	macrophage line J774	6	24 h	• ↓ *Leishmania amazonensis* (intracellular parasite) infection at 5% • ↔ NO or ROS production	(105)

Abbreviations: PBMC, peripheral blood mononuclear cells; con A, concanavalin A; PHA, phytohaemagglutinin; DCF, dichlorofluoroscein; LPS, lipopolysaccharide; GSH, glutathione (reduced); GSSG, glutathione (oxidised); NO, nitric oxide; ROS, reactive oxygen species; HIV, human immunodeficiency virus; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; mTORC, mammalian target of rapamycin, FITC, fluorescein isothiocyanate; IL-12, interleukin 12.

4282 **Table 1-6 The effects of culture at physiological normoxia on intestinal cell physiology**

Cell Type	PO_2 (kPa)	Time (h)	Summary	Reference
T84 Caco-2 B	2.6	0-48	• Intestinal epithelial cells maintain barrier resistance under hypoxia, whereas oral epithelia and endothelial cells increase permeability • ↑ ITF mRNA by 4 h, maintained at protein level for 48 h, and is HIF-1 dependent • Anti-ITF antibody attenuates hypoxia-induced ↑ resistance in Caco-2 cells. Addition of ITF to endothelial cells ↑ resistance during hypoxia. • ITF is induced *in vivo* by hypoxia (8% O_2, 4 h) • Hypoxia *in vivo* associated with increased permeability relative to normoxic controls. Possibly under pathological hypoxia *in vivo*.	(170)
Caco-2	2.6	0-96	• ↑ MRD1 mRNA (18 h) and protein (maintained at 96 h, peak at 48 h) in hypoxia, dependent on HIF-1 • Increase in protein translated to increased surface expression and function (drug efflux)	(93)
T84 Caco-2	2.6	0-96	• ↑ CD79 and CD339 mRNA (18 h) and protein (maintained at 96 h, peak at 48 h) in hypoxia, dependent on HIF-1. • Both proteins are ecto-5'-nucleases responsible for metabolizing 5'-AMP to adenosine, which activates A2B receptors and ↑ barrier integrity	(566)
T84 ModeK	2.6	0-48	• ↑ MUC3 selectively by hypoxia: mRNA (maintained by 24 h) and protein (peaked at 24 h and declined afterwards). Probably HIF-1 mediated. • MUC3 interacts with ITF at the apical surface of epithelial cells	(359)
T84 Caco-2	2	0-48	• Hypoxia ↓ adenosine uptake • ↓ ENT1 and 2 mRNA and protein during hypoxia, maintained at 48 h and HIF-1 dependent • siRNA against ENT2 decreased adenosine uptake, therefore explaining first observation. • Hypoxia *in vivo* associated with increased permeability relative to normoxic controls. Possibly under pathological hypoxia *in vivo*	(408)
T84 Caco-2	2	0-48	• Time-dependent increased Netrin-1 protein during hypoxia, dependent on HIF-1 activity • Endogenous netrin-1 reduces monocyte transepithelial migration via activation of the adenosine A2B receptor on the monocyte. This reduces inflammatory activation and therefore protects the epithelial barrier during inflammation.	(492)
T84 Caco-2	1	0-24	• HIF-1 conditional KO reduced expression of DEFB1 in mouse intestine and Caco-2 cells • Exposure to hypoxia ↑ HIF-1, although basal levels were observed • No change in DEFB1 protein during hypoxia, despite ↑ HIF and dependence on it. • HIF-1 siRNA reduces hBD-1 soluble protein excreted, which would ↑ Bacterial load in intestine • In human bowel scrapings (epithelial-enriched), expression of DEFB1 mRNA correlated linearly with GLUT1 (HIF target), and hBD-1 staining was most prominent at apical edge of colonic mucosa	(293)

Caco-2 T84	1	0-48	• Enzymes involved in creatine metabolism regulated specifically by HIF-2 during hypoxia. • Induction of CKM and CKB maintained at 48 h (protein) and abolished with HIF-2 siRNA • CKM and CKB enzymes are localised to the apical surface of polarised epithelium, and enriched at the adherens junction. • HIF-2 dependent CK induction maintains barrier integrity and protects against inflammation. • Inhibition of CK strongly decreases resistance under hypoxic conditions	(180)
Caco-2	1	0-144	• Measurements of medium PO_2 using optical sensor spots immobilised onto standard 24WPs, gas-permeable 24WP and Transwell inserts • Medium PO_2 reached 4 kPa after 6 days post-confluence in standard 24WP, whereas it was only ~15 kPa in gas permeable plates and in inserts. • Despite low O_2 levels, no HIF-1 stabilisation was observed under standard conditions in normal plates • Medium volume was 1ml: very high for 24-well plate, large depth would lead to O_2 depletion	(690)
Caco-2	1	24	• Hypoxic pre-incubation significantly reduced bacterial internalisation. • This was dependent on β-1 integrin, the protein levels of which were decreased by hypoxic exposure in correlation with increased HIF-1, though no definitive siRNA experiment was performed. • Hypoxia reduces bacterial infection by down-regulating β1 integrin.	(689)

Abbreviations: ITF, Intestinal Trefoil Factor; HIF, hypoxia-inducible factor; MRD1, multidrug resistance protein 1; MUC, mucin; ENT, equilibrative nucleoside transporter; DEFB1; β defensin gene; hBD-1, human β-defensin; GLUT1, glucose transporter 1; CKM/B, creatine kinase M/B;

Table 1-7 The effects of culture at physiological normoxia on bone cell physiology

Species	Cell type	PO_2 (kPa)	Time	Comments	Reference
Human	SV-HFO (human osteoblastic cell line)	2	3 wk	• ↓ mineralisation due to less differentiation and maturation of extracellular matrix components (collagen) • ↓ROS (mitoSOX and DCF) and lower expression of SOD1/2 and CAT	(423)
		2	3 wk	• Microarray of cells cultured at 2 kPa for various phases of differentiation • Impact most prominent during early phase when mineralisation begins to occur	(424)
Mouse	MC3T3-E1 preosteoblast Calvarial organ culture	5	35 d	• Earlier and greater Ca^{2+} mineralisation • ↑ ALP staining and earlier • ↑Osteocalcin expression initially, but declined by 35 d • ↑Cx43 expression at cell-cell junctions and whole cell lysates • ↑Osteocyte markers (Dmp1, Mepe and Fgf23) • ↑osteocyte population (cells expressing Dmp1 and Mepe) in organoid cultures	(241)
	MC3T3-E1 preosteoblast	2, 5	72 h	• 2 kPa strongly associated with HIF stabilisation (PMZD staining and HRE luciferase), not observed at 5 kPa • ↑PGE_2 production and EP1 receptor expression at 2 kPa	(329)
	MLOA5 (preosteoblast) MLOY4 (osteocyte)	2, 5	7 d	• ↓PO_2 associated with ↓ALP activity and mineral deposition • no change in viability • ↑HIF stabilisation at 2 kPa preserves cell function under hypoxia	(686)
Rat	Calvarial osteoblasts	10	7 d	• Compared to hyperoxia (90% O_2), ↑proliferation • No change in ALP activity	(595)
	Primary osteoblasts	0.2-20	18 d	• ↓bone module formation with ↓PO_2 • at 2 kPa O_2, ↓proliferation but no change in apoptosis • Delayed osteoblast differentiation markers (osteocalcin and PLOD) and ↓collagen production.	(601)
Rabbit	Periosteal organ culture	1-90	6 wk	• Highest growth and chondrogenesis at 15 kPa • ↓collagen synthesis at very high and very low O_2 levels • Culture in a 'sealed 'jar' apparatus	(435)

Abbreviations: ALP, alkaline phosphatase; PLOD, procollagen lysine, 2-oxoglutarate, 5-dioxygenase; Cx43, connexin 43; Dmp1, dentin matrix protein 1; Mepe, matrix extracellular phosphoglycoprotein; Fgf23, fibroblast growth factor 23; PMZD, pimonidazole; HRE, hypoxia-responsive element; PGE_2, prostaglandin E_2; EP1, prostaglandin E receptor 1; HIF, hypoxia-inducible factor; ROS, reactive oxygen species; DCF, dichlorofluorescein; SOD, superoxide dismutase; CAT, catalase; SV-HFO, SV40-transformed Human Fetal Osteoblastic cells

4293 **Table 1-8 The effects of culture at physiological normoxia on chondrocyte physiology**

Species	Cell Type	PO_2 (kPa)	Time	Comments	Reference
Human	Chondrocytes	5	4 wk	• ↑ collagen II, aggregan and SOX9 mRNA in 3D culture • ↑ proliferation and GAG synthesis • no change in pH or glucose consumption • Redifferenatiated phenotype only achieved when 5 kPa O_2 and 3D culture combined	(414)
	Meniscus cells Chondrocytes	5	2 wk	• no change in collagen I mRNA • ↑ Collagen II and SOX9 mRNA in meniscus cells but not chondrocytes	(4)
		5	3 wk	• ↑ GAG and hyaline-cartilage like matrix deposition • ↑ Collagen II, aggregan and SOX9 protein expression	(287)
		5	5 d	• no change in aggregan or Col I mRNA expression, or in DNA content, lactate production or glucose consumption • ↑ VEGF production and mRNA levels at pH 7.4 and 5 kPa O_2	(104)
		5	4 wk	• no change in growth or DNA content • ↑ CD44 and CD105 expression • ↑ Pellet volume and GAG content • no change in col1/2 mRNA expression, but ↑ aggregan mRNA	(518)
		20	NA	• Modeling O_2 consumption and PO_2 profiles in 3D alginate chondrocyte cultures • PO_2 <8 kPa makes chondrocytes favor proteoglycan synthesis over collagen	(341)
Bovine	Chondrocytes	3-60	6 d	• ↓ DNA content with ↓ PO_2 • ↓ Sulphate incorporation, as indicative of GAG synthesis, with ↓ PO_2 • ↑ Proteoglycan aggregation with ↓ PO_2	(86)
		0.1, 5, 10, 20	7 d	• no change in viability or proliferation, which didn't actually occur • no change in aggregan expression • ↑ pellet volume peaking at 10 kPa O_2, indicative of ↑ collagen synthesis • no change in pH until 0.1 kPa • ↓ rRNA yield at 0.1 kPa. Suggested that chondrocytes are particularly robust in withstanding hypoxia relative to other cell types	(196)
		5	4 wk	• no change in growth, viability or pH • no change in Collagen I mRNA • ↑ Collagen II and aggregan mRNA • ↑ matrix collagen and GAG content during 3D culture. • Culture at 5 kPa exacerbated redifferentiation during 3D culture • All culture performed on orbital shakers so pericellular PO_2 was also ~5 kPa	(415)

Bovine		5	3 wk	• ↓ Collagen I mRNA during redifferentiation • ↑ Collagen II, XI and aggregan mRNA during redifferentiation • ↑ Collagen staining in 3D beads, especially towards the periphery where PO_2 would be highest	(117)
		5	3 wk	• ↓ Collagen I and ↑ Collagen II/aggregan mRNA and staining during redifferentiation • ↑ sulfate and proline incorporation, indicative of GAG and proteoglycan synthesis	(118)
		2, 5	14 d	• ↓ proliferation at both O_2 levels but no change in protein content • ↑ O_2 consumption during monolayer culture which was proportional to PO_2 • ↑ ROS at 20 kPa • Differences in cellular phenotype between 2 and 5 kPa	(240)
		2, 5	9 d	• ↑ mitochondrial mass during culture , more pronounced at 20 kPa than 2. Associated with ↑ oxidative phosphorylation • ↑ ROS and O_2 consumption at 20 kPa which could be restored to fresh/ 2 kPa values but treatment with NAC • ↑ GAG accumulation in 3D culture at 5 kPa	(239)
Mouse	C3H10T1/2 E14.5 murine forearm limb	5	7 d	• ↑ BMP-2 induced GAG synthesis with concomitant ↓ ALP activity and alizarin red staining, indicating less osteoblastic differentiation. • No change in adipocyte differentiation • No change in proliferation • ↑ Col2a1 mRNA expression and ↓ Col10a1 mRNA expression, mediated by ↑ p-p38 and HDAC4 activity. • Enlarged matrix area in forelimbs cultured at 5 kPa O_2 • 5 kPa O_2 promotes chondrogenic differentiation but not terminally.	(242)
Rabbit	Chondrocyte	5	3 wk	• ↑ proliferation • ↑ Collagen II mRNA but no change in aggregan or collagen I • ↑ aggregation with more collagen and GAG deposition. • Culture at 5 kPa O_2 during the expansion phase more pronounced than during the 3D phase	(233)

Abbreviations: GAG, glycosaminoglycan; ROS, reactive oxygen species; NAC, N-acetyl cysteine; BMP-2, bone morphogenic protein 2; ALP, alkaline phosphatase; ColX, collagen X; HDAC4, histone deacetylase 4; VEGF, vascular endothelial growth factor.

4297 **Table 1-9 The effects of culture at physiological normoxia on marrow-derived stem cells**

Species	Cell type	PO_2 (kPa)	Time	Comments	References
Human	MSC	1	1-3 wk	• ↓proliferation • ↓adipogenic and chondrogenic differentiation	(251)
	MSC in pellet culture	2	14 d	• ↑sulphated GAG, collagen and proteoglycan formation, with more homogenous surface distribution. More chondrogenic	(374)
	haemopoietic	5	10 d	• ↑CD34+ CD38- expansion due to ↑proliferation, not ↓apoptosis • ↑myeloid-committed cells (CD33+ CD14+) with ↑colony forming potential • ↑subsequent cell engraftment in irradiated mice	(495)
	MSC	5, 2	thawing and duration of culture	• ↓colony numbers and cell yield at 5% specifically, but ↓colony and individual cell size at 2%. • ↑bone-marrow specific markers (CD44, 73 and 105) at both 5 and 2 kPa • ↓cellular senescence at 5 and 2% • ↓O_2 consumption (microelectrode) and ↑lactate production, with ↓proportion of ATP generated by oxidative phosphorylation (azide sensitive) • ↑ chondrogenic differentiation at 5% specifically	(449)
	Haemopoietic (CD44+ progenitor)	5	>7 d	• ↓NO production (Griess) • ↑proliferation and colony formation, with ↑progenitor cell production and proliferation	(483)
	mesenchymal	3	up to 100 d	• ↑colony formation following isolation at 3 kPa • ↔ hypoxia-associated genes, but 2313 differentially expressed genes related mostly to ageing (↓) and bone-marrow specific markers (↑) • ↓senescence over 100 d, with high population doubling times • ↓AGE accumulation and SA-β-gal+ cells, markers of senescence	(149)
	MIAMI	3	15 d	• highest proliferation at 3 kPa • ↑embryonic and 'stem' genes OCT4, REX-1 and hTeRT • ↓osteoblast marker genes Runx and OCN • ↓ALP activity, mineral deposition and ECM-associated Ca^{2+} content • MIAMI cells less differentiation/mature than MSC.	(101)
	Marrow nucleated cells	1,5,10	6 d	• Largest colonies formed with the highest density of cells at 5 kPa • Similar colonies formed between 1 and 20 kPa	(616)
Porcine	MSC	5	42 d	• Agarose constructs contained increased GAG deposition, particularly within the core. • ↑Col2a1 and ↓Col1a1 relative to room air/	(391)

	MSC	5	expd/dif/both	• expansion at 5% results in larger colony formation and thus higher cell yield • Differentiation at 5%, even when following expansion at 20%, enhances the chondrogenic potential of MSC; more stable pellet/hydrogel with ↑Ca^{2+} content and ↓collagen X	(528)
Rat	MSC	5	up to 21 d	• Enhanced calcium content and mineralisation at 5 kPa O_2 suggests cells more likely differentiate in response to stimulation at 5 kPa O_2	(336)
Sheep	mesenchymal	5	up to 14 d	• ↑colony size and number, ↑proliferation • ↓senescence • ↑chondrogenic differentiation	(700)

Abbreviations: GAG, glycosaminoglycan; ColX, collagen X; MSC, mesenchymal stem cell; NO, nitric oxide; SA-β-gal, senescence associated β-galatosidase; AGE, advanced glycosylation end-products; OCT4, octamer-binding transcription factor 4; hTeRT, human telomerase reverse transcriptase; Runx; Runt-related transcription factor 1; OCN, osteocalcin; ALP, alkaline phosphatase; ECM, extracellular matrix; MIAMI, Marrow-isolated adult multilineage inducible cells; MSC, mesenchymal stem cells

Table 1-10 The effects of culture at physiological normoxia on skin cell physiology

Species	Cell Type	PO_2 (kPa)	Time (d)	Comments	Reference
Human (Mouse)	82-6 and Bj (HCA2) MEF	3	60 (from isolation)	• MEF cultures did not undergo senescence at 3 kPa, but it could be induced by exposure to 20 kPa at early time points • At 3 kPa, MEFs behaved more like human fibroblasts • Changes in expression patterns of p53 regulatory proteins $p19^{ARF}$ and p16 at 3 kPa. • ↑ Chromosomal breakage at 20 kPa in MEFs • MEF are particularly sensitive to oxygen metabolism damage, but at 3 kPa are just as robust as human fibroblasts	(446)
	Keratinocyte	4	From isolation	• Comparison between cells from young and old donors • ↑migration (scratch) in young, but not old, cells. No change in proliferation • ↓ROS (DHE) independent of age • Scratching induces ROS production at leading edge, dissipation of which related to migration rate	(493)
	Dermal Fibroblasts	2	>3 d	• No change in metabolic activity or morphology • ↓ inhibition of metabolic activity by blue light (450 nm) at 2 kPa, whereas NIR light (850 nm) stimulated metabolism only at 2 kPa	(394)
	HCT116 Keratinocytes	5	4	• ↑ HIF protein at 5 kPa in HCT116, much less in keratinocytes (barely visible) Accompanied by HRE-Luc activity and GLUT-1 mRNA increases in HCT116 cells • Inhibition of MAPK cascade with U0126 reduced HIF1 protein, as did treatment with doxorubicin • ↑ proliferation in HCT116 cells at 5 kPa, barely effected by HIF1 KO. • All observations consistent with 'self-inflicted hypoxia' due to O_2 consumption. Inhibition of MAPK or DNA damaging agents would reduce cellular activity (O_2 consumption), thereby increasing pericellular	(68)
Mouse	Tail fibroblasts MEF	3	7.5	• Spontaneous chromosomal breaks at 20 kPa reduced at 3 kPa in cells with compromised chromosome stability ($Ku86^{-/-}$) • No change in cell proliferation • Some level of spontaneous instability even at 3 kPa, probably due to other sources of ROS	(283) (78)
Rodent	Fibroblasts	3	30 d	• Fibroblasts from lab mouse strains are the only rodent cells to exhibit marked sensitivity to O_2	(448)

Abbreviations: MEF, mouse embryonic fibroblasts; ROS, reactive oxygen species; DHE, dihydroethidium; HRE-Luc, luciferase conjugated hypoxia-responsive element; GLUT-1, glucose transporter 1; MAPK, mitogen activated protein kinase

Table 1-11 The effects of culture at physiological normoxia on adipose tissue resident cells

Species	Cell type	PO_2 (kPa)	Time (d)	Comments	Reference
Human	Adipocytes	15, 10, 5, 3, 1	1	• Leptin, VEGF and IL-6 mRNA and secretion inversely proportional to PO_2 • Adiponectin mRNA and secretion proportional to PO_2 • Steepest part of sigmoid curves between 3-10 kPa, with 10 kPa often not different from room air • Glucose uptake and GLUT transporter expression increased proportional to PO_2 and corresponds to increased lactate secretion	(663)
	Adipocytes	10, 5	14	• No change in differentiation (Oil red O staining), but decreased lipid droplet size proportional to PO_2 • Increased triglyceride content at 10 kPa • Increased expression and secretion of adiponectin, IL-6 and DPP4, with concomitant decreases in leptin secretion (adipokine secretion) • Increased glycerol release and hormone sensitive lipase expression at 10 kPa (lipolysis) • Conditioned medium from 10 kPa cells induced the lowest SMC proliferation	(145)
	Adipose MSC	5	duration of induction	• a combination of kinase chemical inhibitors and 5% O_2 is optimal for the induction of iPSCs from adipose-derived stem-cells	(533)
	Adipose MSC	2	duration of culture	• ↑proliferation • ↑viability • ↑adipogenic and osteogenic differentiation potential following pre-adaptation to low O_2	(606)
	Adipose MSC	5	21	• ↓fatty acid binding protein 4 expression • ↓glycerol-3-phosphate dehydrogenase activity and triglyceride content, markers of adipogenic differentiation • effects of low O_2 abolished by cytoskeletal inhibitors cytochalasin D and blebbistatin	(512)
Human Rabbit	Adipose stromal cells	5	21	• culture at 5 kPa increases chondrogenic differentiation of stromal cells *in vitro* but does not increase subsequent chondrogenesis *in vivo*	(464)

Abbreviations: VEGF, vascular endothelial growth factor; IL-6, interleukin-6; GLUT, glucose transporter; DPP4; Dipeptidyl peptidase-4; SMC, smooth muscle cell; iPSC, inducible pluripotent stem cell; MSC, mesenchymal stem cell

Table 1-12 The effects of physiological normoxia on embryonic stem cell physiology

Species	Cell type	PO_2 (kPa)	Time (d)	Comments	Reference
Human	ESC (H1)	5	12-15	• hESC differentiation in culture was inversely proportional to PO_2: little differentiation apparent when cultured at 1-4 kPa O_2 • Greater embryonic body formation under low O_2 • Phenomenon is not reversible, exposure to room air after low O_2 does not induce much more differentiation	(142)
	ESC (H1, RH1 and H9)	2	14	• Clonal recovery was 4-12-fold higher at 2 kPa and cells were less differentiated • Switching back to room air reduced colony formation • Less spontaneous chromosomal alterations and perhaps longer telomeres	(161)
	ESC (H1, RH1 and H9)	2	10 passages	• Genetic screen of cells cultured for 10 passages at 2 or 18 kPa O_2 • Between p0 and 10, no change in genome at room air, but robust genomic changes at 2 kPa	(160)
	H1 and H9	4	7 (after multiple passages)	• Cultures at 4 kPa displayed less heterogeneity in their transcriptome • No change in the expression of pluripotency after culture at 20 kPa for 7 days, although a number of genes under the OCT4/NANOG/SOX2 triad are expressed much less	(643)
	CLS1 / 2	1, 5, 10, 15	Up to 8 weeks	• Colony formation as inversely proportional to PO_2 • After 4 weeks, re-exposure to room air rapidly induced proliferation and differentiation, even in cultures kept at 5 kPa for 18 months • Colonies at 5 kPa O_2 homogenous and undifferentiated • Inhibition of notch signaling normalised differentiation	(469)
	Blastocyst-derived	5	From thawing	• Cells thawed at 5 kPa O_2 and then exposed to room air for >3 d • No gross changes in genome after chronic culture at room air • Spontaneous differentiation reduced after exposure to room air • Exposure to room air induces irreversible X-chromosome inactivation	(335)
	H9	5	6-12	• At room air, dissolved PO_2 ~15 kPa, and near 1 kPa at 5 kPa ambient • Decreased O_2 consumption at 5 kPa • Early vascular markers (CD42, KDR and CD56) increased at 5 kPa, with morphological evidence for increased endothelial differentiation • When differentiation was split into 2 parts; culture at 5 kPa was only required for the first 6 days • Culture at 5 kPa increases ROS formation (DCF) and inhibition with DPI abolished this and the increased EC differentiation	(320)
	MEL-2 ESC	2	Up to 5	• No significant change in proliferation • ↑ Lactate and glucose consumption, and ↓ ammonia consumption	(596)

				• ↑ production of alanine, and ↑ consumption of aspartate, asparagine and glycine. ↓ consumption of glutamine • Good information on O_2 diffusion and limitations *in vitro*. Intracellular PO_2 most certainly close to 0 kPa at 2 kPa ambient in ESC cultures	
Human	Shef3 and Hues-7	5	Minimum of 3 passages	• No large change in amino acid utilisation or production • ↑ Glycine consumption • ↓ serine uptake	(82)
	hESC and hiPSC	2	>5	• Genomic screen suggested culture at 2 kPa shifted cells towards neuronal lineages (expression of SOX1, HES5 and FOXG1), specifically towards glial differentiation vs neuronal • Effect was abolished by HIF-2 siRNA and HIF-a inhibition • HIF regulates MYC and thus LIN28/let-7, a key neuronal lineage regulator	(666)
	Blastocyst-derived	5	From derivation/ thawing	• Imprinted region DLK1-DIO3 frequently silenced during culture due to hyper-methylation • When lines were derived and maintained at 5 kPa, gene products from this regions (MEG3 and SNORD114-3) were still highly expressed • Re-exposure to room air caused rapid downregulation of these gene products and methylation of their promoters • Greater genomic stability when cultured and maintained at 5 kPa O_2	(665)
	Shef3 and Hues-7	5	Minimum of 3 passages	• ↑ GLUT3 expression and membrane localisation • GLUT3 mRNA positively correlates with OCT4 mRNA • Changes in GLUT3 expression may underlie changes in lactate/glucose consumption	(83)
Mouse	D3	4	9	• mESC cultured at 4 kPa for 9 days and then back to room air for 5 more. Early culture at 4 kPa O_2 increased differentiated cardiomyocyte yield	(30)
	stem cell (fresh)	5	28	• More outgrowths from blastocysts and increased proliferation with more alkaline phosphatase activity in outgrowths	(176)
	stem cell (fresh)	5	from blastocyst isolation	• ↑overall cellular yield • ↓ROS production (measured using DCF fluorescence) • combination of low O_2 and physiological levels of glucose (100mg/dL) optimum for yield	(629)
	CCE and D3 mESC	0, 1, 5, 20 and 40	Up to 25	• Mathematical modelling of PO_2 at the cell layer • ↑ proliferation at 5 kPa vs room air • Very low O_2 levels (<1 kPa) ↓ pluripotency genes (Oct4, Nanog, Sox2) and at 40 kPa	(467)
	D3 mESC	3.5	12	• Greater degree of differentiation into dopaminergic neuronal precursor cells at low O_2 and with greater yield	(300)

Mouse	iPSC (MEF)	5	up to 30	• ↑efficiency of pluripotency induction and ↑yield. Observed with both viral and non-viral transfection methods • no change in proliferation • ↑57.2% ESC-specific genes, ↓67.5% fibroblast-specific genes. Demonstrates efficiency in differentiation	(678)
	R1	1, 2, 4, 8	7	• Production and secretion of Flt-1 and VEGF change temporally over 7 days and at low O_2, driving ESC into either hematopoietic or endothelial lineages at different time points • Colony formation greatest at 2-4 kPa O_2 and lowest at 1 and 20 kPa	(473)
	E14Tg2a mESC	2	8	• Increased viability during 8 days of differentiation • ↑ neuronal stem cell differentiation. • Valuable data on dissolved PO_2 in culture	(404)
	E14Tg2a mESC	2	8	• Highest number and diameter of neural bodies (rosettes) between 4-10 kPa O_2, with the greatest number of viable cells • Greatest number of neuronal precursor cells observed at 4 kPa O_2 • Increases from 2-4/6 kPa O_2 associated with ↑ total yield	(403)
	R1 129/Sv (ESC) MAR B6D2 F1 (ESC)	5	3	• ↑apoptosis • ↑proliferation • ↑ROS generation (measured using DCF fluorescence) • ↓cardiomyocyte differentiation	(475)
	E14Tg2A (ESC) iPSwt4F (iPSC)	5	up to 12	• ↑embryonic body formation • ↑expression of NKx2.1 and FOXA2, markers of endodermal and early lung epithelial lineages • ↑differentiation into early pulmonary lineages following stimulated differentiation	(173)

Abbreviations: ESC, embryonic stem cell; ROS, reactive oxygen species; DCF, dichlorofluoroscein; OCT4, octamer-binding transcription factor 4; Flt-1, vascular endothelial growth factor receptor 1; KDR, kinase insert domain receptor/vascular endothelial growth factor receptor 2; DPI, diphenyleneiodonium; EC, endothelial cell; NKx2.1, NK2 Homeobox 1; FOXA4, forkhead box protein A2; iPSC, inducible pluripotent stem cell; HES5, Hes Family BHLH Transcription Factor 5; FOXG1, forkhead box protein G1; HIF, hypoxia-inducible factor; MYC, v-Myc avian myelocytomatosis viral oncogene homolog; DLK1-DIO3, delta-like homolog 1- type III iodothyronine deiodinase gene cluster; GLUT, glucose transporter.

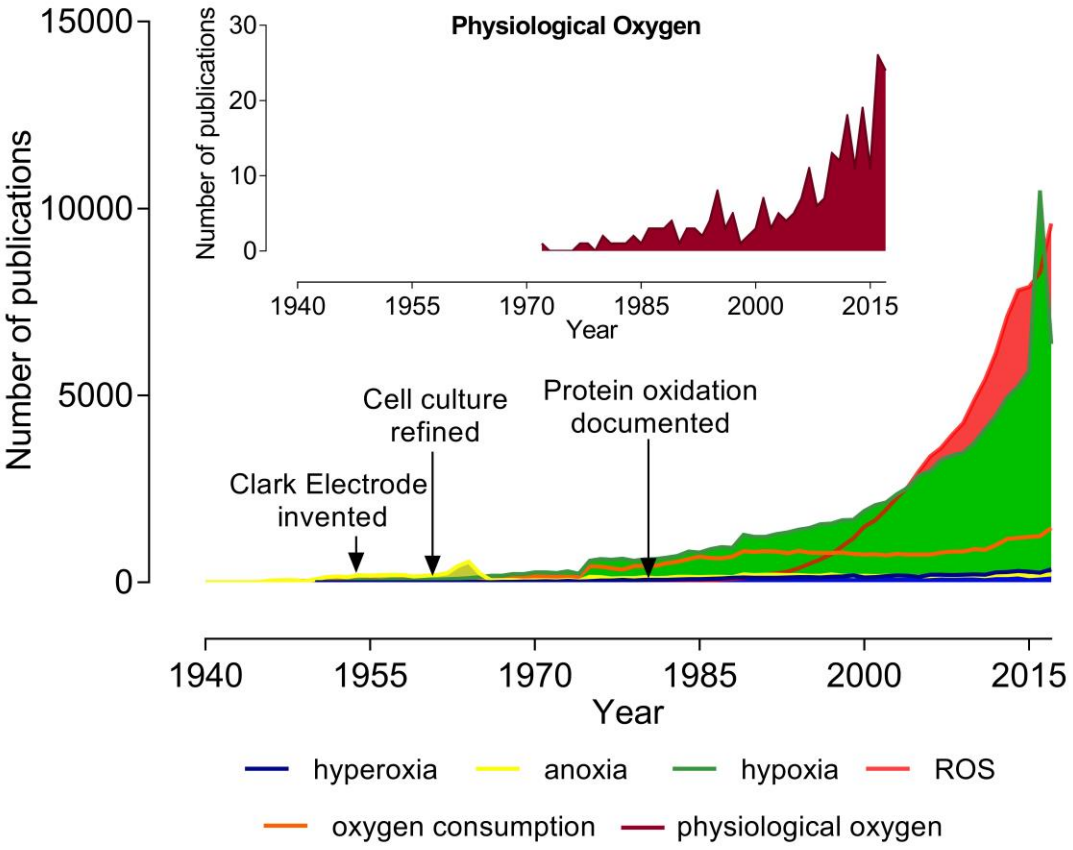

Physiological Oxygen
Number of publications
30
20
10
0
1940
1955
1970
1985
2000
2015
Year
15000
10000
5000
0
Number of publications
Cell culture refined
Protein oxidation documented
Clark Electrode invented
1940
1955
1970
1985
2000
2015
Year
hyperoxia
anoxia
hypoxia
ROS
oxygen consumption
physiological oxygen

Figure 1

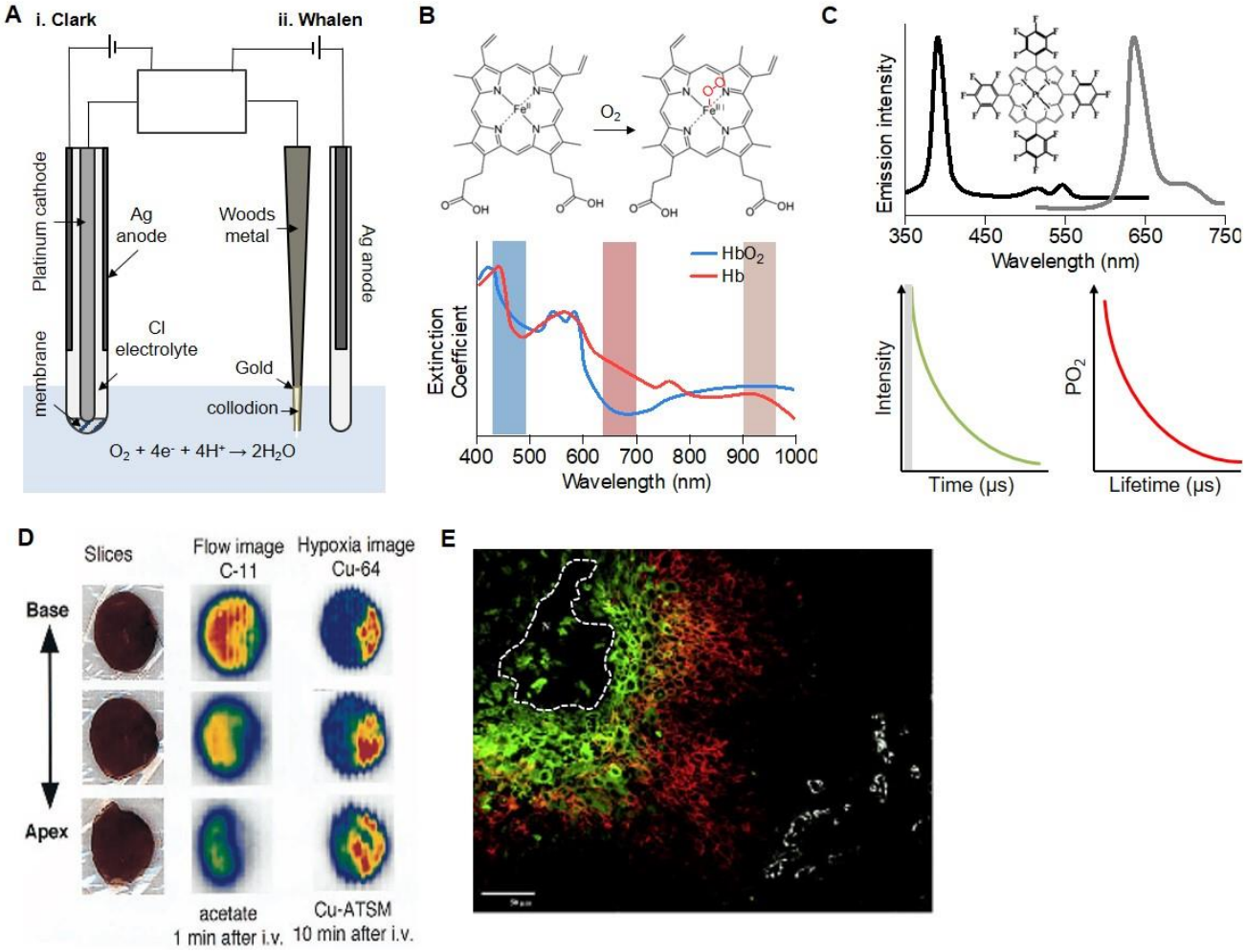

A
i. Clark
ii. Whalen
Platinum cathode
Ag anode
Woods metal
Ag anode
membrane
Cl electrolyte
Gold
collodion
$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$
B
O_2
Extinction Coefficient
HbO_2
Hb
400 500 600 700 800 900 1000
Wavelength (nm)
C
Emission intensity
350 450 550 650 750
Wavelength (nm)
Intensity
Time (μs)
PO_2
Lifetime (μs)
D
Slices
Flow image C-11
Hypoxia image Cu-64
Base
Apex
acetate
1 min after i.v.
Cu-ATSM
10 min after i.v.
E

Figure 2

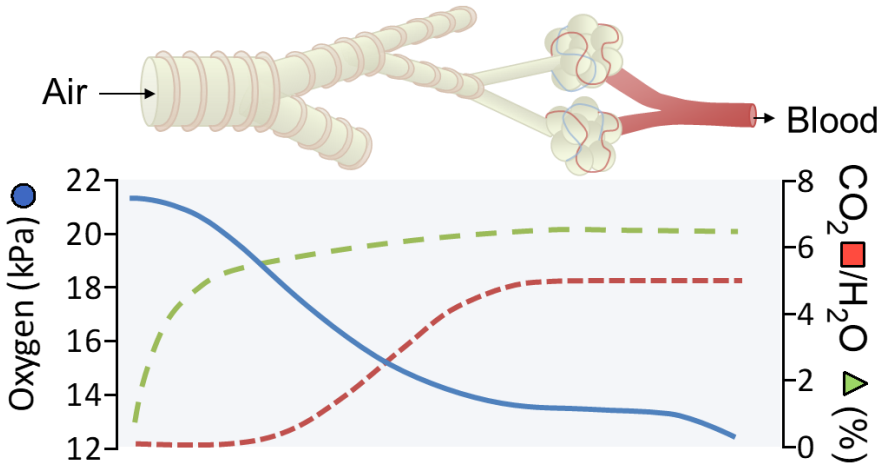


Figure 3

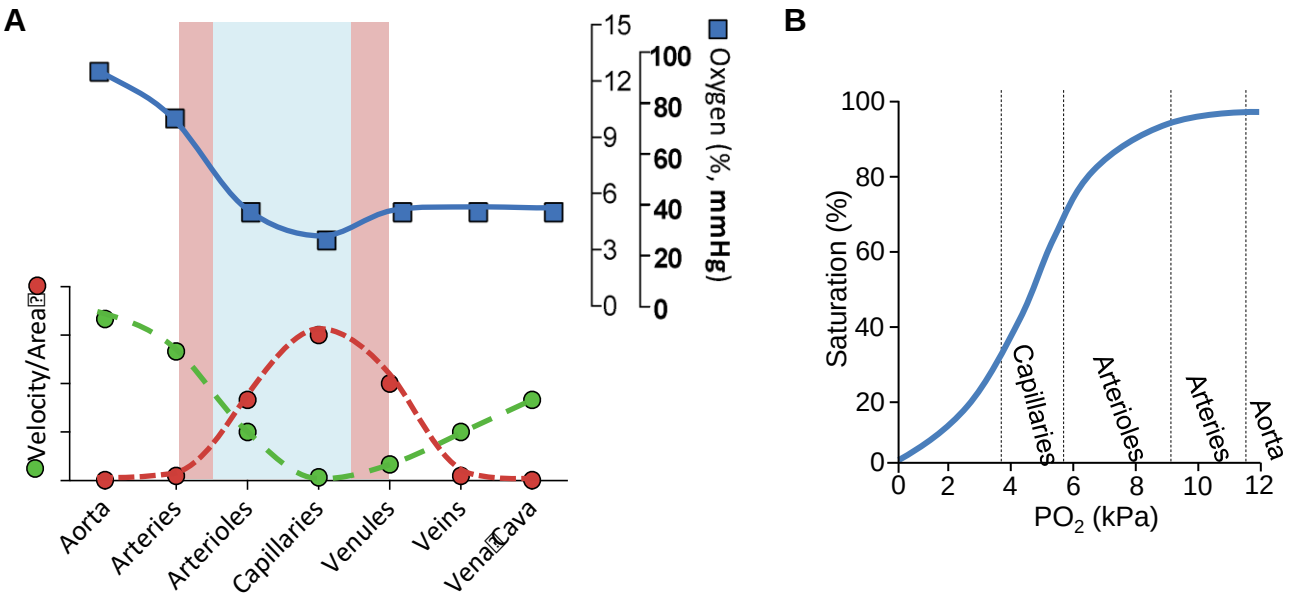

A
Velocity/Area
Oxygen (%, mmHg)
15
12
9
6
3
0
100
80
60
40
20
0
Aorta
Arteries
Arterioles
Capillaries
Venules
Veins
Vena Cava
B
Saturation (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
PO2 (kPa)
Capillaries
Arterioles
Arteries
Aorta

Figure 4

A

	%	kPa	mmHg	µM
%	-	1.02	7.65	13.5
kPa	0.98	-	7.5	13.2
mmHg	0.13	0.132	-	1.76
µM	0.074	0.076	0.57	-

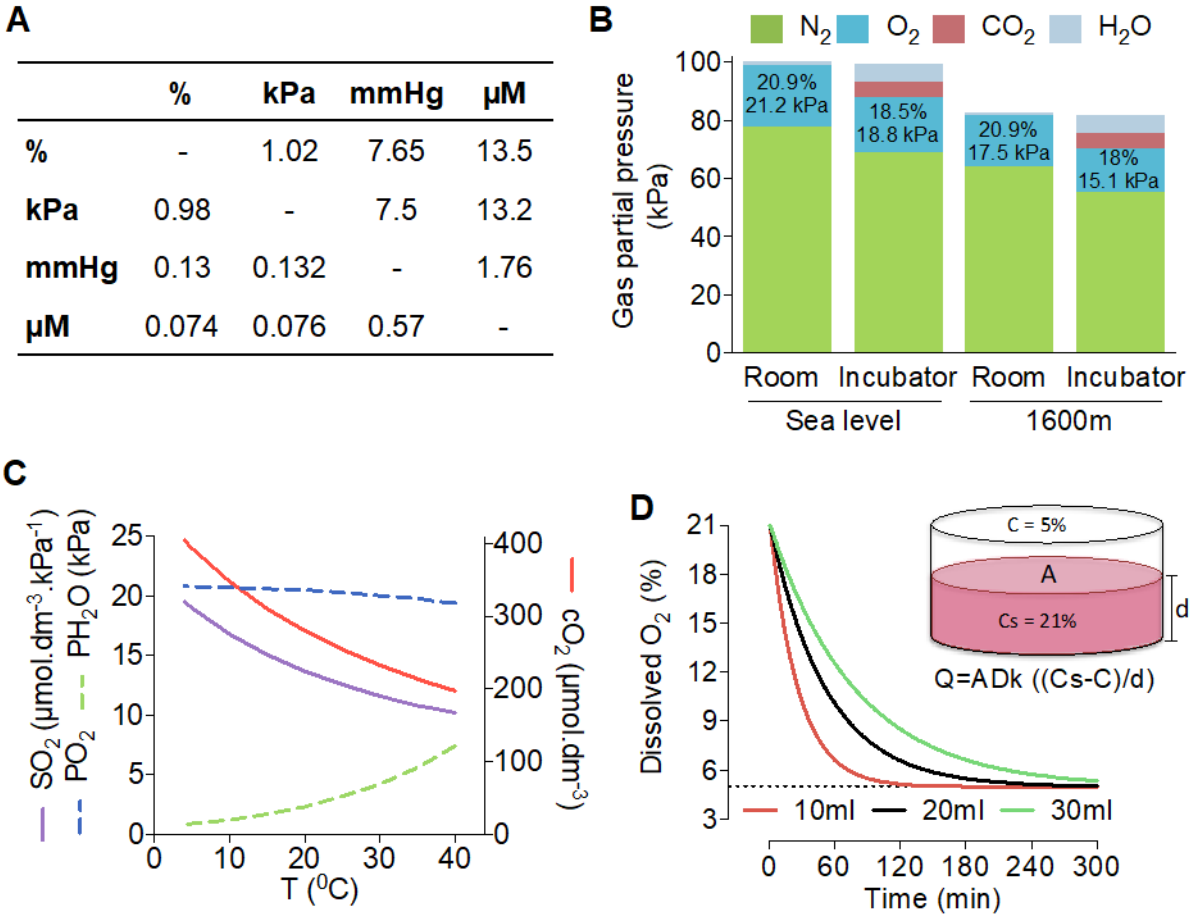


Figure 5

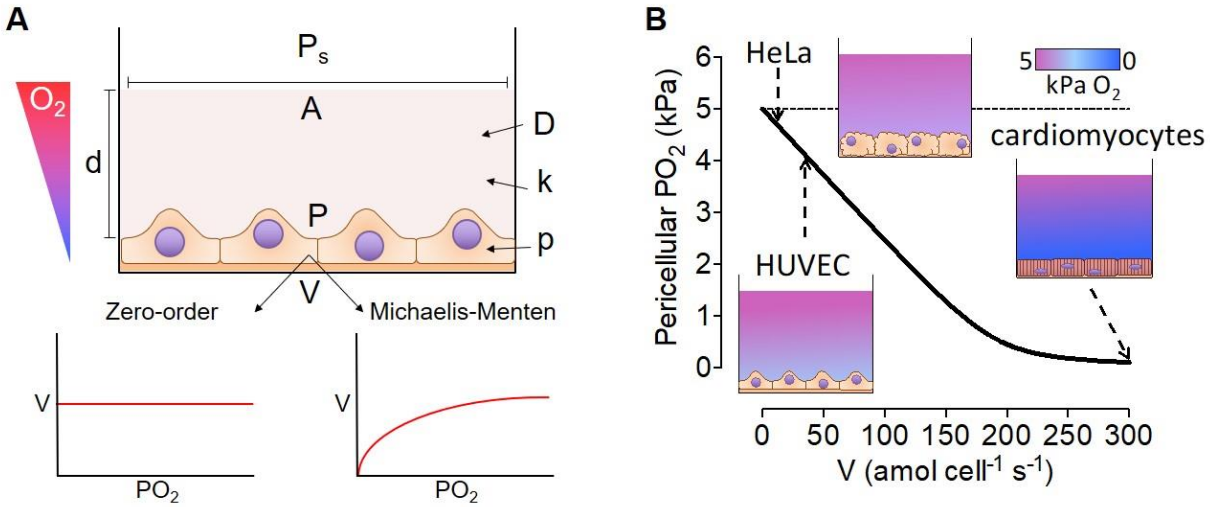

A
P_s
O_2
A
d
D
k
P
p
V
Zero-order
Michaelis-Menten
V
PO_2
V
PO_2
B
HeLa
5
0
kPa O_2
cardiomyocytes
HUVEC
Pericellular PO_2 (kPa)
6
5
4
3
2
1
0
0 50 100 150 200 250 300
V (amol cell^{-1} s^{-1})

Figure 6

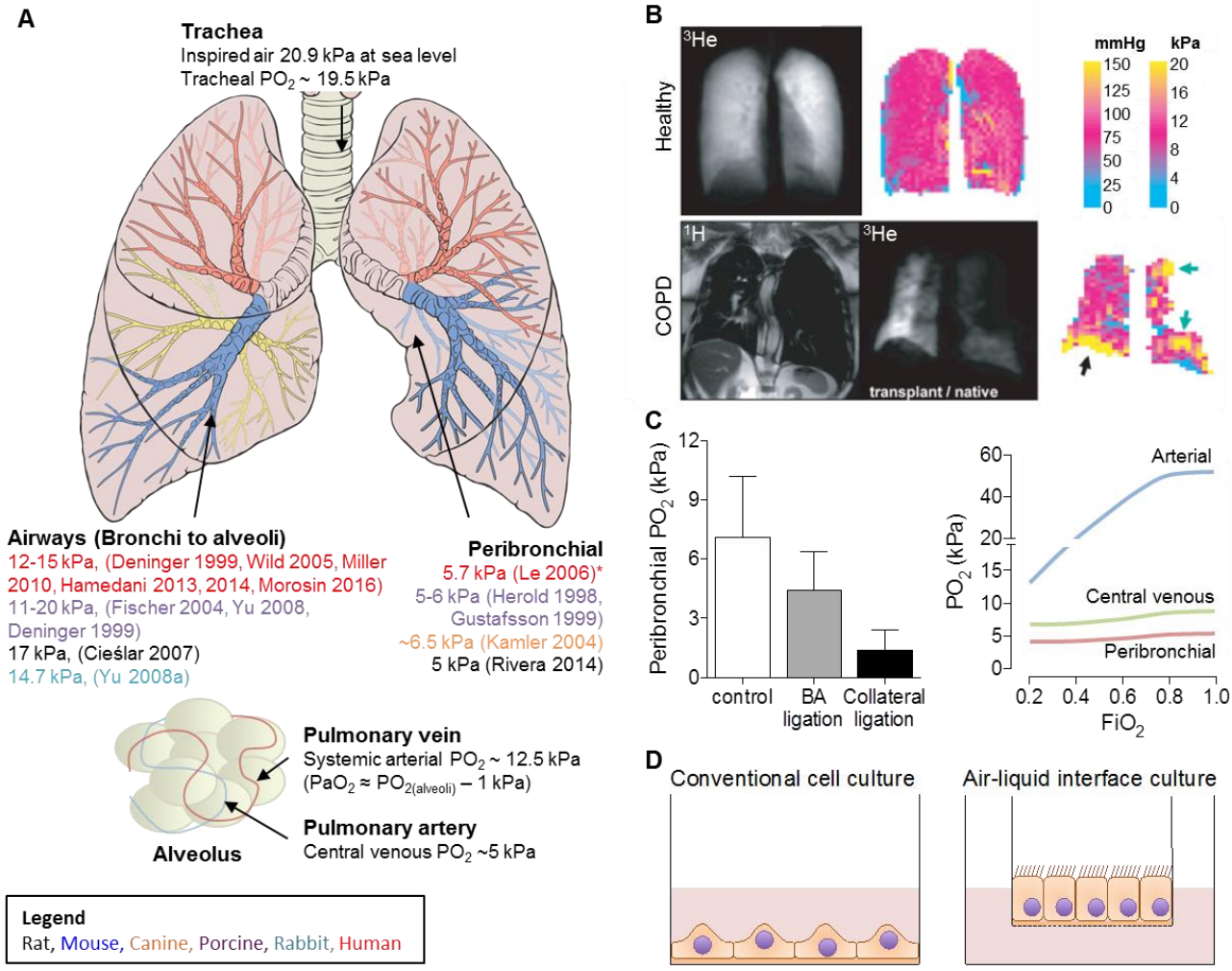
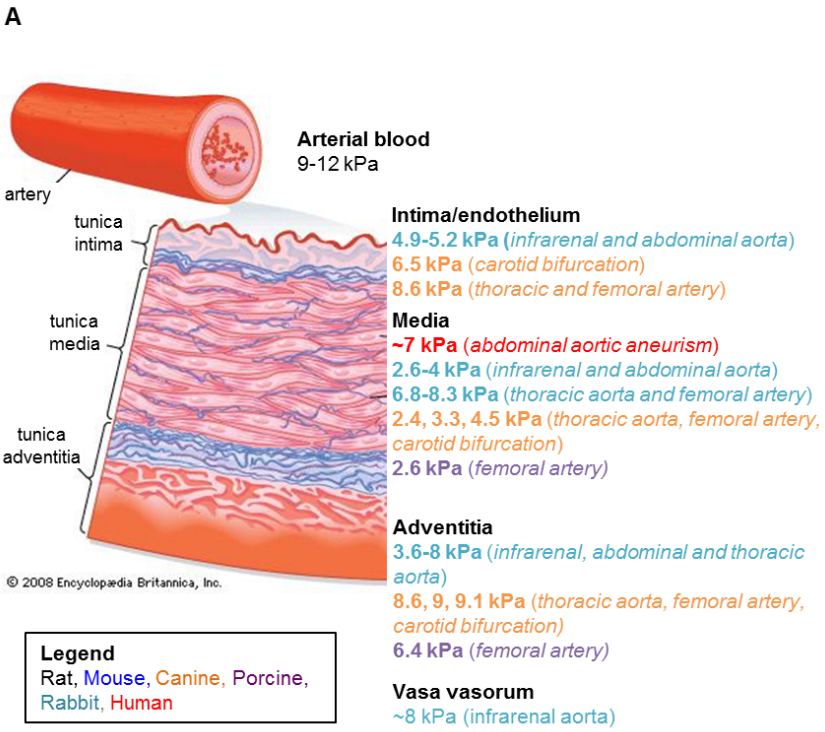

A
Trachea
Inspired air 20.9 kPa at sea level
Tracheal PO_2 ~ 19.5 kPa
Airways (Bronchi to alveoli)
12-15 kPa, (Deninger 1999, Wild 2005, Miller 2010, Hamedani 2013, 2014, Morosin 2016)
11-20 kPa, (Fischer 2004, Yu 2008, Deninger 1999)
17 kPa, (Cieślar 2007)
14.7 kPa, (Yu 2008a)
Peribronchial
5.7 kPa (Le 2006)*
5-6 kPa (Herold 1998, Gustafsson 1999)
~6.5 kPa (Kamler 2004)
5 kPa (Rivera 2014)
Pulmonary vein
Systemic arterial PO_2 ~ 12.5 kPa
($PaO_2 \approx PO_{2(alveoli)}$ – 1 kPa)
Pulmonary artery
Central venous PO_2 ~5 kPa
Alveolus
Legend
Rat, Mouse, Canine, Porcine, Rabbit, Human
B
Healthy
COPD
^3He
^1H
transplant / native
mmHg
kPa
C
Peribronchial PO_2 (kPa)
control
BA ligation
Collateral ligation
PO_2 (kPa)
Arterial
Central venous
Peribronchial
FiO_2
D
Conventional cell culture
Air-liquid interface culture

Figure 7


A
artery
Arterial blood
9-12 kPa
tunica intima
tunica media
tunica adventitia
© 2008 Encyclopædia Britannica, Inc.
Intima/endothelium
4.9-5.2 kPa (infrarenal and abdominal aorta)
6.5 kPa (carotid bifurcation)
8.6 kPa (thoracic and femoral artery)
Media
~7 kPa (abdominal aortic aneurism)
2.6-4 kPa (infrarenal and abdominal aorta)
6.8-8.3 kPa (thoracic aorta and femoral artery)
2.4, 3.3, 4.5 kPa (thoracic aorta, femoral artery, carotid bifurcation)
2.6 kPa (femoral artery)
Adventitia
3.6-8 kPa (infrarenal, abdominal and thoracic aorta)
8.6, 9, 9.1 kPa (thoracic aorta, femoral artery, carotid bifurcation)
6.4 kPa (femoral artery)
Vasa vasorum
~8 kPa (infrarenal aorta)
Legend
Rat, Mouse, Canine, Porcine, Rabbit, Human

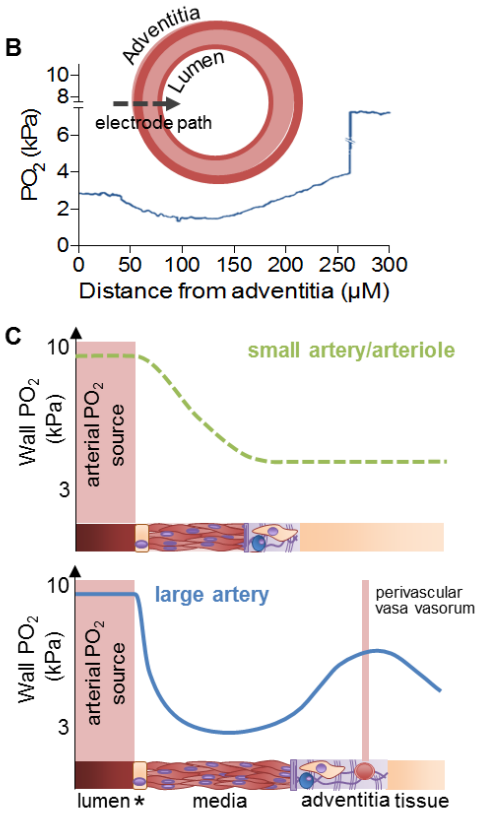

B
Adventitia
Lumen
electrode path
PO2 (kPa)
10
8
6
4
2
0
0 50 100 150 200 250 300
Distance from adventitia (μM)
C
small artery/arteriole
Wall PO2 (kPa)
10
3
arterial PO2 source
large artery
perivascular vasa vasorum
lumen
media
adventitia tissue

Figure 8

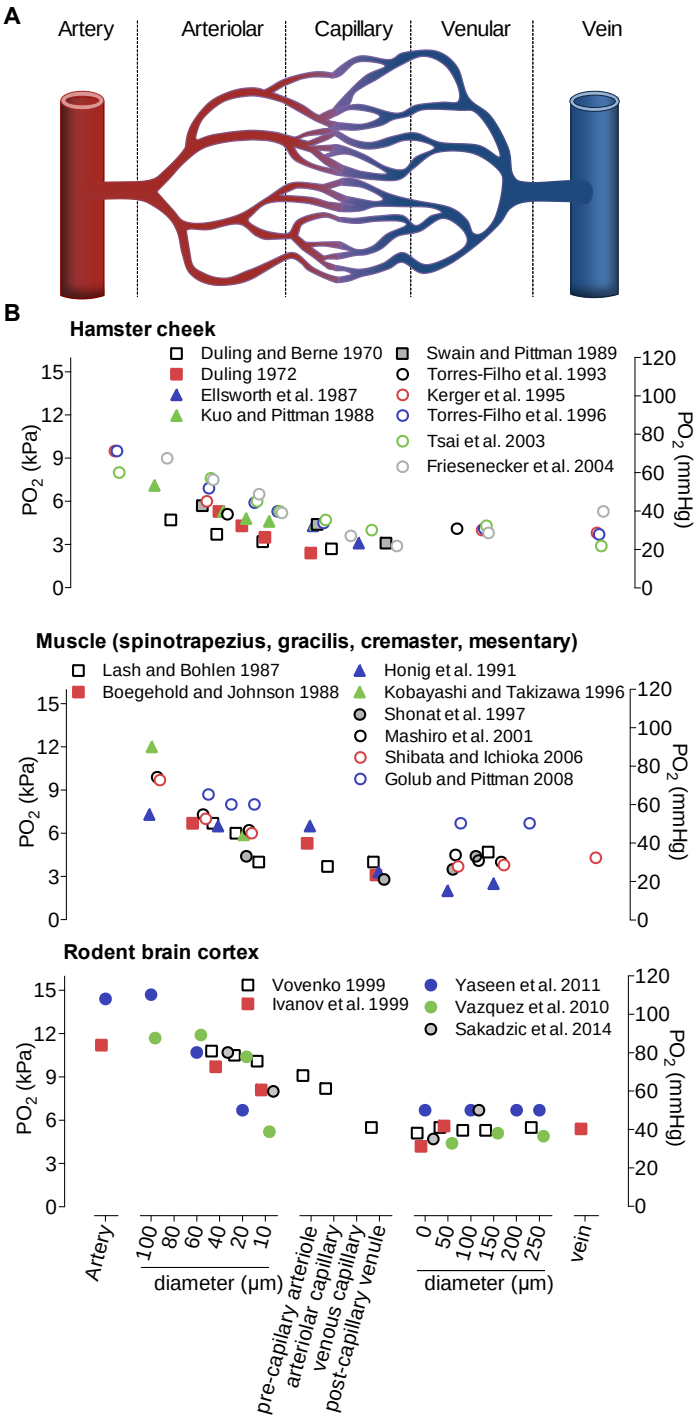

A
Artery
Arteriolar
Capillary
Venular
Vein
B
Hamster cheek
Duling and Berne 1970
Duling 1972
Ellsworth et al. 1987
Kuo and Pittman 1988
Swain and Pittman 1989
Torres-Filho et al. 1993
Kerger et al. 1995
Torres-Filho et al. 1996
Tsai et al. 2003
Friesenecker et al. 2004
PO2 (kPa)
PO2 (mmHg)
Muscle (spinotrapezius, gracilis, cremaster, mesentary)
Lash and Bohlen 1987
Boegehold and Johnson 1988
Honig et al. 1991
Kobayashi and Takizawa 1996
Shonat et al. 1997
Mashiro et al. 2001
Shibata and Ichioka 2006
Golub and Pittman 2008
Rodent brain cortex
Vovenko 1999
Ivanov et al. 1999
Yaseen et al. 2011
Vazquez et al. 2010
Sakadzic et al. 2014
Artery
diameter (μm)
pre-capilary arteriole
arteriolar capillary
venous capillary
post-capillary venule
vein

Figure 9

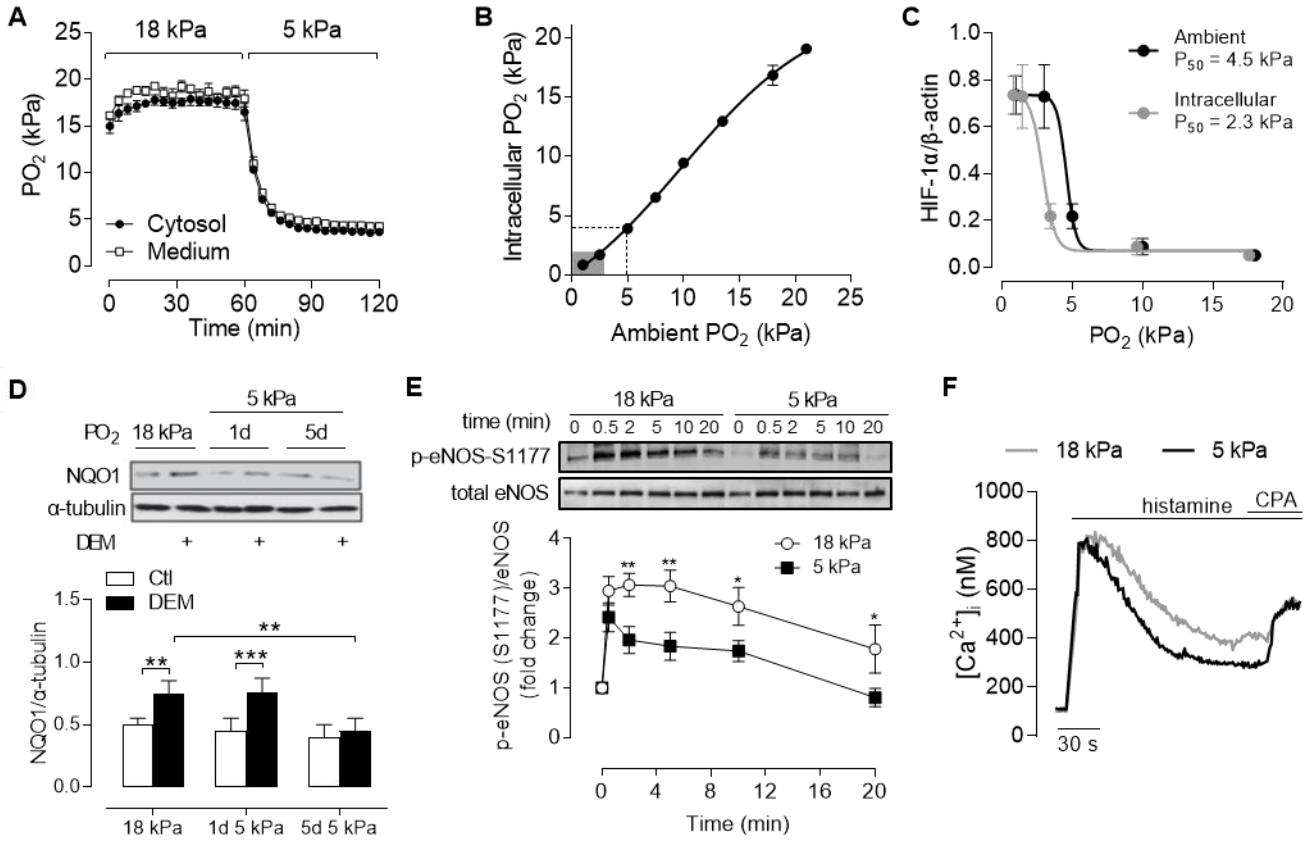
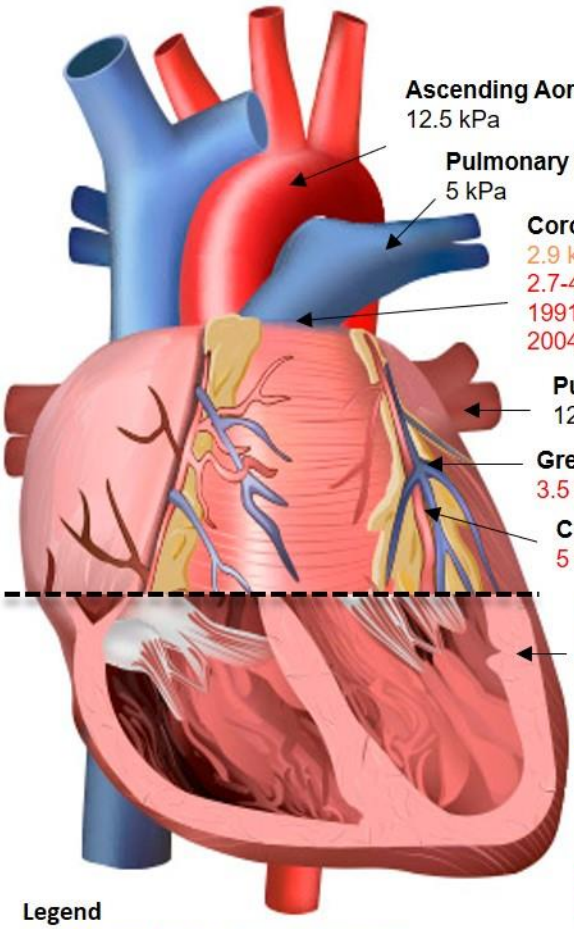

A
18 kPa
5 kPa
PO2 (kPa)
Cytosol
Medium
Time (min)
B
Intracellular PO2 (kPa)
Ambient PO2 (kPa)
C
Ambient
P50 = 4.5 kPa
Intracellular
P50 = 2.3 kPa
HIF-1α/β-actin
PO2 (kPa)
D
5 kPa
PO2 18 kPa 1d 5d
NQO1
α-tubulin
DEM + + +
Ctl
DEM
NQO1/α-tubulin
18 kPa
1d 5 kPa
5d 5 kPa
E
time (min)
18 kPa
5 kPa
0 0.5 2 5 10 20 0 0.5 2 5 10 20
p-eNOS-S1177
total eNOS
p-eNOS (S1177)/eNOS (fold change)
18 kPa
5 kPa
Time (min)
F
18 kPa
5 kPa
histamine
CPA
[Ca2+]i (nM)
30 s

Figure 10



Ascending Aorta
12.5 kPa

Pulmonary artery
5 kPa

Coronary sinus
2.9 kPa (Case et al. 1975)
2.7-4.5 kPa (White et al. 1991, Vretzakis et al. 2004, McNulty et al. 2005)

Pulmonary vein
12.5 kPa

Great cardiac vein
3.5 kPa (Wilkinson et al. 1980)

Coronary artery
5 kPa (Gundry et al. 1996)

Myocardium
1.9-3.4 kPa (Winbury et al. 1971, Angell et al. 1975, Lösse et al. 1975, Feola et al. 1979)
2-5 kPa (Khan et al. 2008, Li et al. 2010)
2.6-2.9 kPa (Chacko et al. 2009, Rivera et al. 2014)
4.1 kPa (Skolasinska et al. 1978)
2-3.3 kPa (Wiener et al. 1982, Bjerrum et al. 2006)
6.1 kPa (Hobbhahn et al. 1989)

Legend
Rat, Mouse, Feline, Canine, Porcine, Rabbit, Human

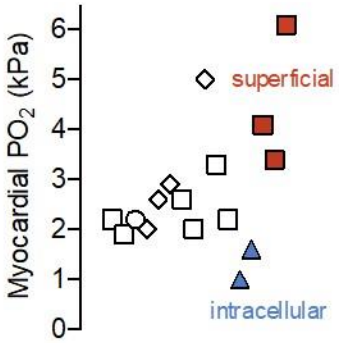
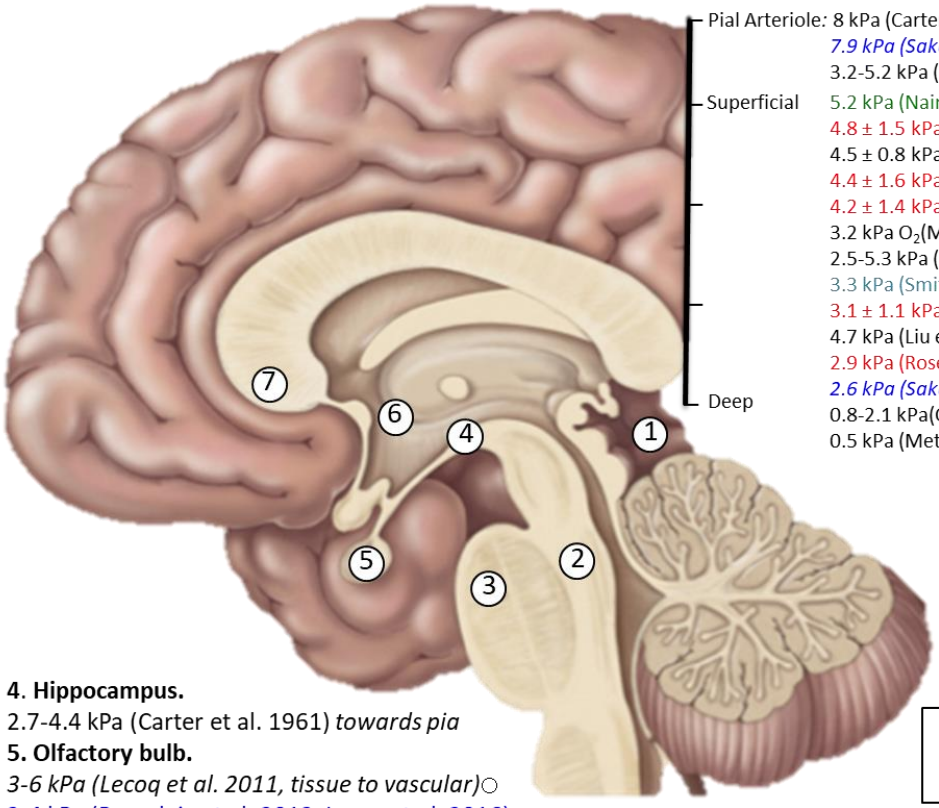


Figure 11



Pial Arteriole: 8 kPa (Carter et al., 1961)
7.9 kPa (Sakadzic et al. 2010) ○
3.2-5.2 kPa (Metzger and Heuber 1977)

Superficial
5.2 kPa (Nair et al., 1975)
4.8 ± 1.5 kPa (Hoffman et al. 1996)
4.5 ± 0.8 kPa (Liu et al., 1995)
4.4 ± 1.6 kPa (Ding et al., 1998)
4.2 ± 1.4 kPa (Hoffman et al. 1997)
3.2 kPa O_2(Metzger and Heuber 1977
2.5-5.3 kPa (Carter et al., 1961)
3.3 kPa (Smith et al., 1977)
3.1 ± 1.1 kPa (Ding et al. 1998)
4.7 kPa (Liu et al. 1995) ⍰
2.9 kPa (Rosenthal et al., 2008)
2.6 kPa (Sakadzic et al., 2010) ○

Deep
0.8-2.1 kPa(Carter et al., 1961)
0.5 kPa (Metzger and Heuber 1977)

1. Ventricle.
5.5 kPa O_2 (Zauner et al. 1995)

4. Hippocampus.
2.7-4.4 kPa (Carter et al. 1961) *towards pia*
5. Olfactory bulb.
3-6 kPa (Lecoq et al. 2011, tissue to vascular)○
3-4 kPa (Parpaleix et al. 2013, Lyons et al. 2016)
6. Hypothalamus. 1.5-2.1 kPa (Carter et al. 1961)
7. Caudate nucleus. 0.8-3.5 kPa (Seylaz and Pinard, 1978)

Legend
Rat, Mouse, Feline, Rabbit, Human

2. Midbrain. 0.6-117 kPa (Carter et al., 1961)
3. Pons. 0.1-0.4 kPa (Carter et al., 1961)

Figure 12

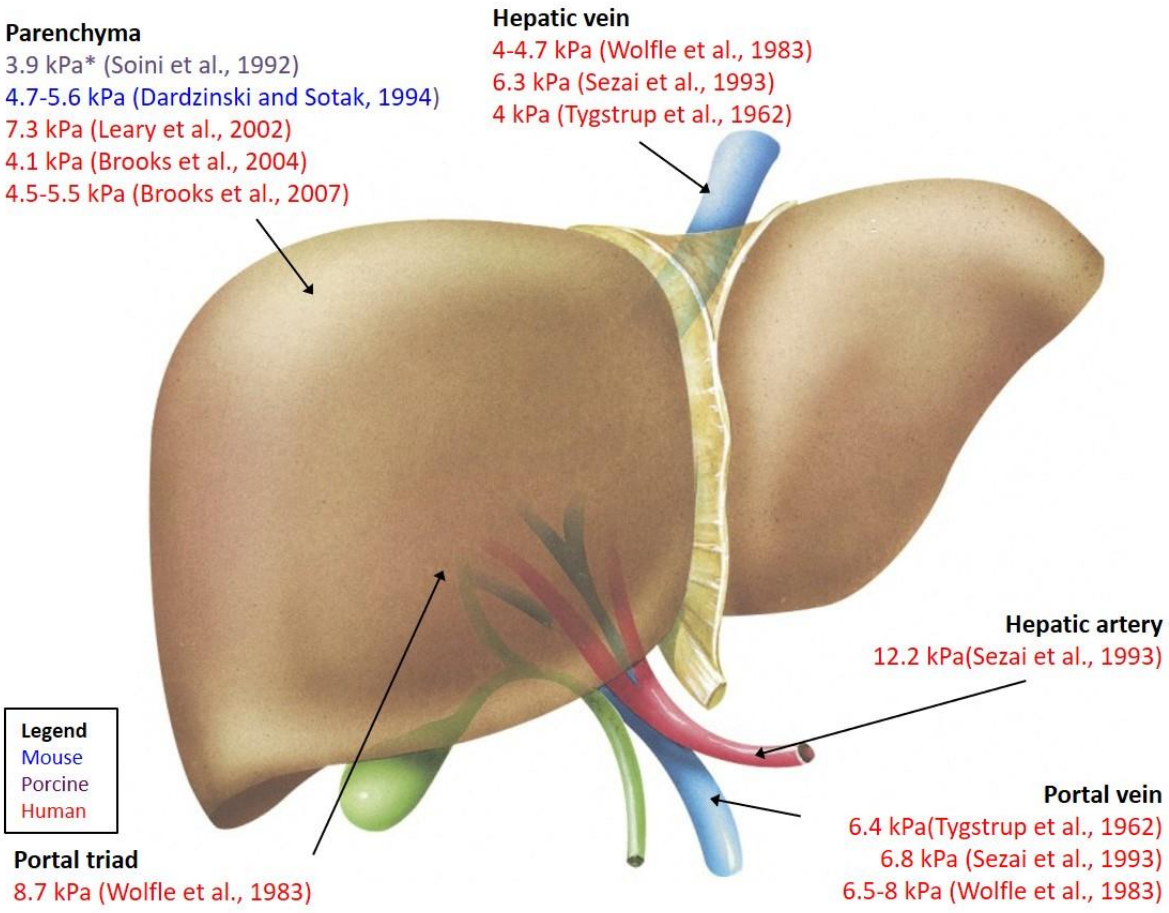

Parenchyma
3.9 kPa* (Soini et al., 1992)
4.7-5.6 kPa (Dardzinski and Sotak, 1994)
7.3 kPa (Leary et al., 2002)
4.1 kPa (Brooks et al., 2004)
4.5-5.5 kPa (Brooks et al., 2007)
Hepatic vein
4-4.7 kPa (Wolfle et al., 1983)
6.3 kPa (Sezai et al., 1993)
4 kPa (Tygstrup et al., 1962)
Hepatic artery
12.2 kPa(Sezai et al., 1993)
Portal vein
6.4 kPa(Tygstrup et al., 1962)
6.8 kPa (Sezai et al., 1993)
6.5-8 kPa (Wolfle et al., 1983)
Legend
Mouse
Porcine
Human
Portal triad
8.7 kPa (Wolfle et al., 1983)

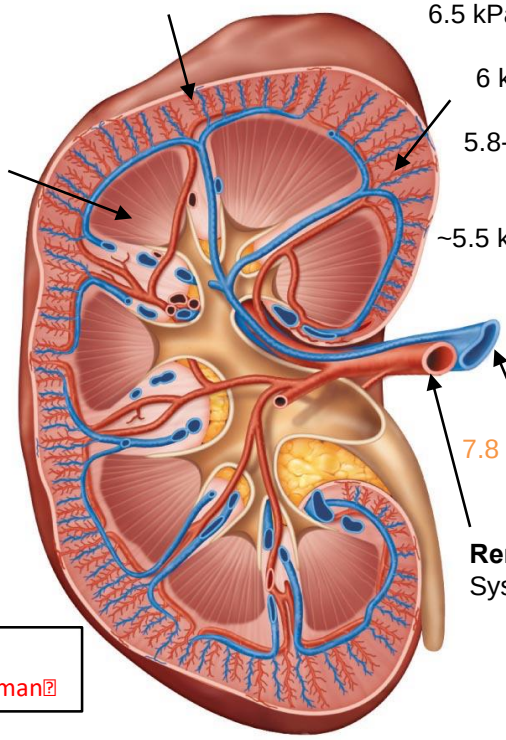
Figure 13

Surface
4.8 kPa (Singawitz et al., 1978)
4 kPa (Singawitz et al., 1978)
5.8 kPa (Lent et al., 1982)
9.4 kPa (Müller et al., 1998)

Cortex
~6.5 kPa (0-3 mm depth), (Leichtweiss et al., 1969)
4.7 kPa (Baumgärtl et al., 1972)
5 kPa (Günther et al., 1974)
6.5 kPa @ 1.6mm depth (Brezis et al., 1991)
6 kPa @superficial glomerulus (Schurek et al., (1990)
5.8-7.2 kPa @1.5mm (Brezis et al., 1994)
5.8 kPa (Liss et al., 1996)
~5.5 kPa (Lübbers and Baumgärtl 1997)
6 kPa (Zhang et al., 2014)

Medulla
~1.3 kPa (>3 mm depth), (Leichtweiss et al., 1969)
1-1.3 kPa (Baumgärtl et al., 1972)
1.3 kPa (Günther et al., 1974)
3.5 kPa @ 4.2mm depth (Brezis et al.,1991)
1.3-2.3 kPa @4.5mm (Brezis et al., 1994)
4.2 - 3.3kPa (outer to inner) (Liss et al., 1996)
~1.3 kPa (Lübbers and Baumgärtl 1997)
3.3 kPa (Zhang et al., 2014)
2 kPa (Zhang et al., 2014)

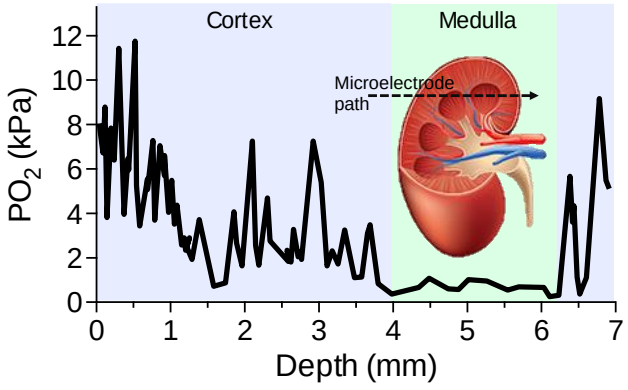
Tubules
5.2 kPa (Proximal + Distal), (Welch et al., 2001)



Renal Vein
6.5 kPa (Welch et al., 2001)
7.8 kPa (Gullichsen et al., 1989)

Renal Artery
Systemic arterial =12.5 kPa

Legend
Rat, Canine, Porcine, Rabbit, Human



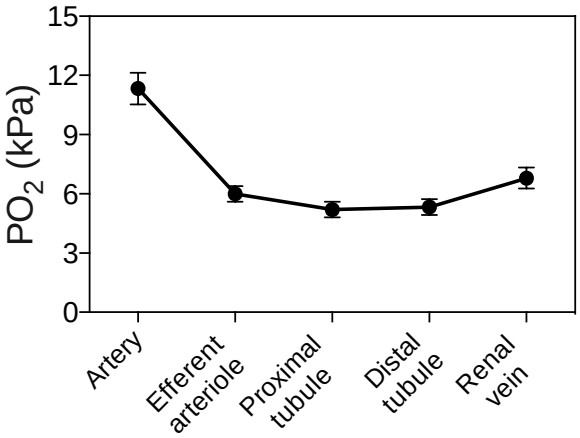


Figure 14

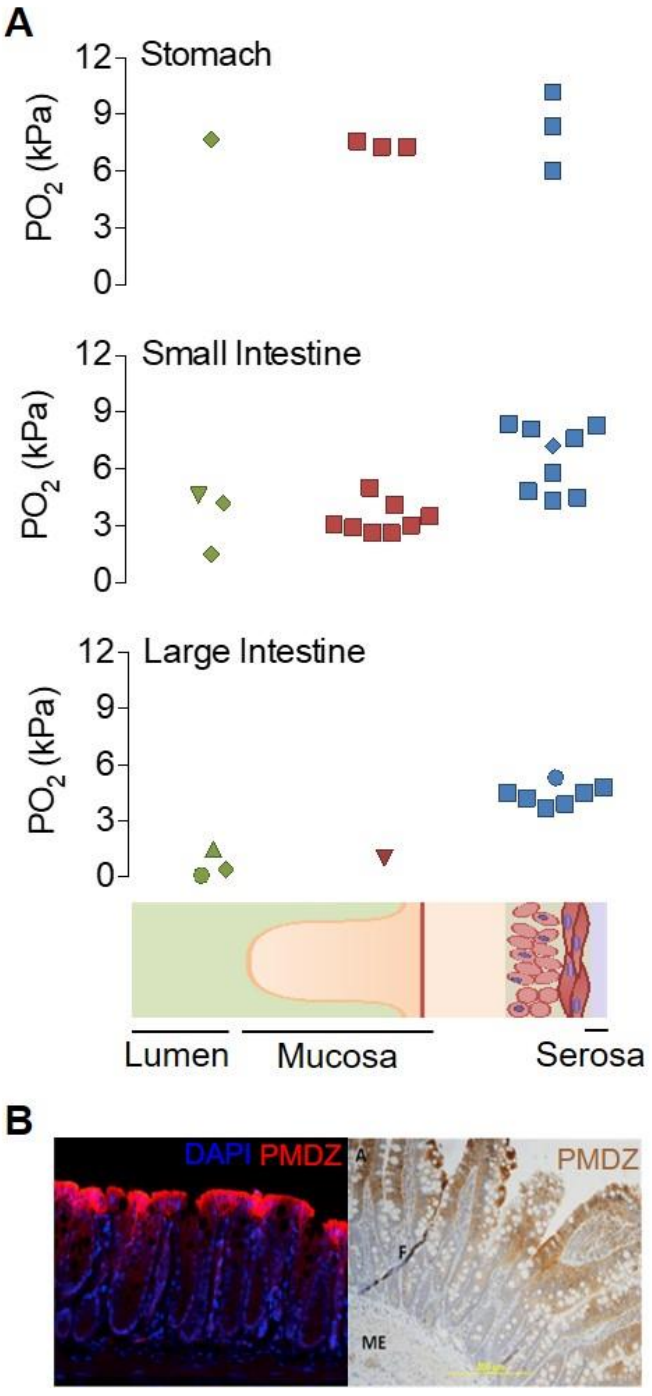

A
Stomach
Small Intestine
Large Intestine
PO2 (kPa)
12
9
6
3
0
Lumen
Mucosa
Serosa
B
DAPI PMDZ
PMDZ
A
F
ME

Figure 15

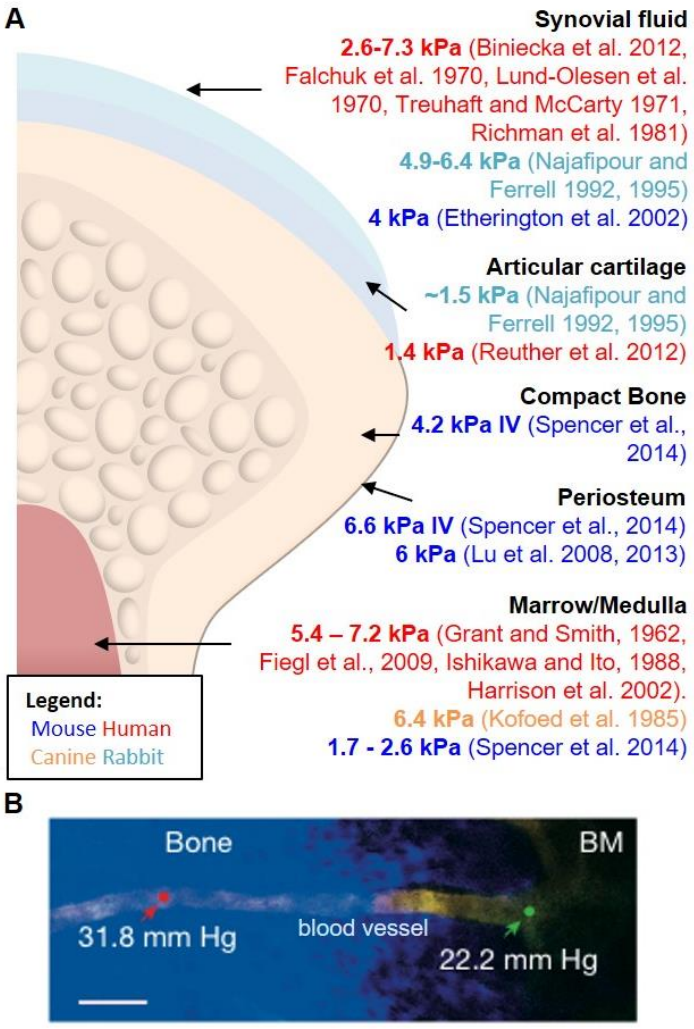

A
Synovial fluid
2.6-7.3 kPa (Biniecka et al. 2012, Falchuk et al. 1970, Lund-Olesen et al. 1970, Treuhaft and McCarty 1971, Richman et al. 1981)
4.9-6.4 kPa (Najafipour and Ferrell 1992, 1995)
4 kPa (Etherington et al. 2002)
Articular cartilage
~1.5 kPa (Najafipour and Ferrell 1992, 1995)
1.4 kPa (Reuther et al. 2012)
Compact Bone
4.2 kPa IV (Spencer et al., 2014)
Periosteum
6.6 kPa IV (Spencer et al., 2014)
6 kPa (Lu et al. 2008, 2013)
Marrow/Medulla
5.4 – 7.2 kPa (Grant and Smith, 1962, Fiegl et al., 2009, Ishikawa and Ito, 1988, Harrison et al. 2002).
6.4 kPa (Kofoed et al. 1985)
1.7 - 2.6 kPa (Spencer et al. 2014)
Legend:
Mouse Human
Canine Rabbit
B
Bone
BM
31.8 mm Hg
blood vessel
22.2 mm Hg

Figure 16

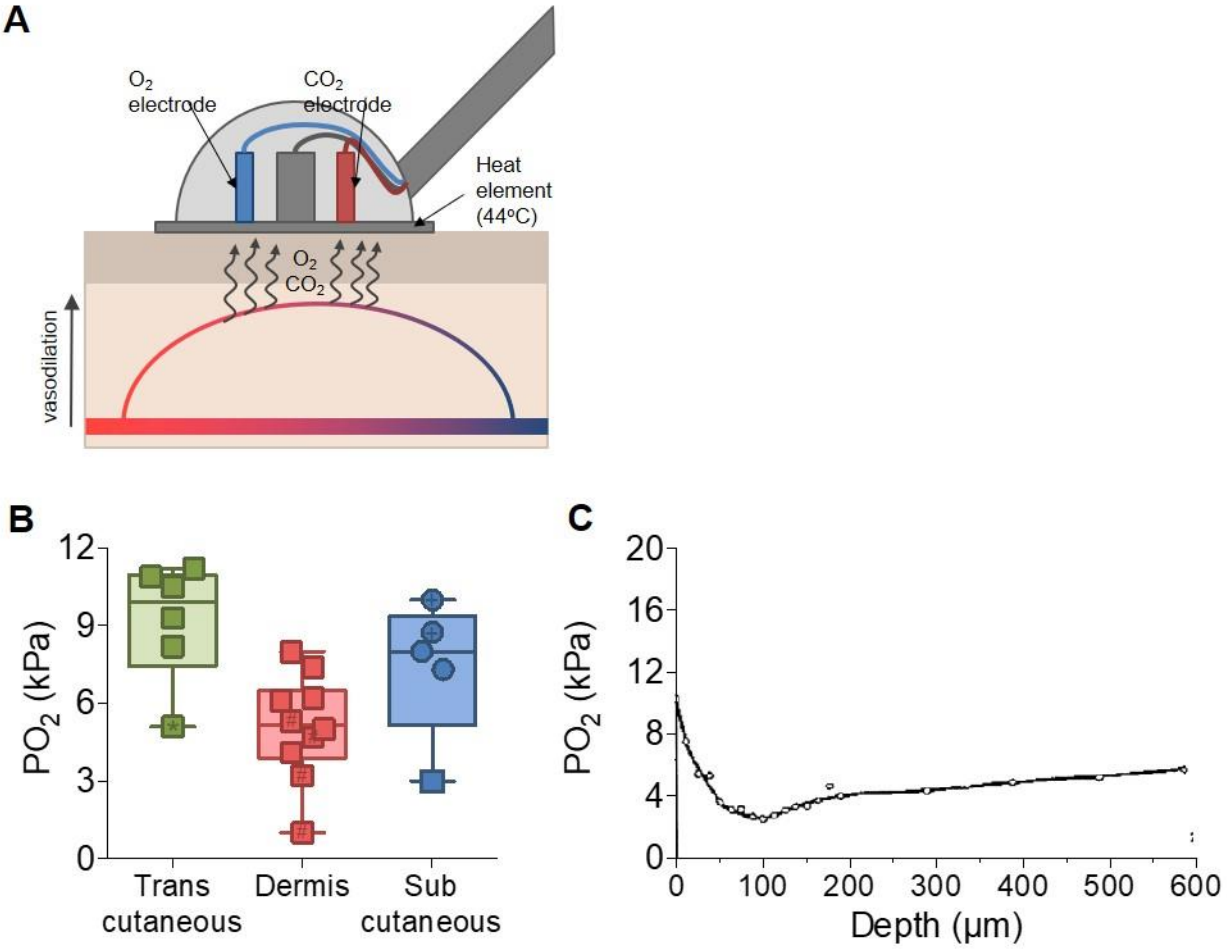

A
O_2 electrode
CO_2 electrode
Heat element (44°C)
O_2
CO_2
vasodilation
B
PO_2 (kPa)
12
9
6
3
0
Trans cutaneous
Dermis
Sub cutaneous
C
PO_2 (kPa)
20
16
12
8
4
0
0
100
200
300
400
500
600
Depth (µm)

Figure 17

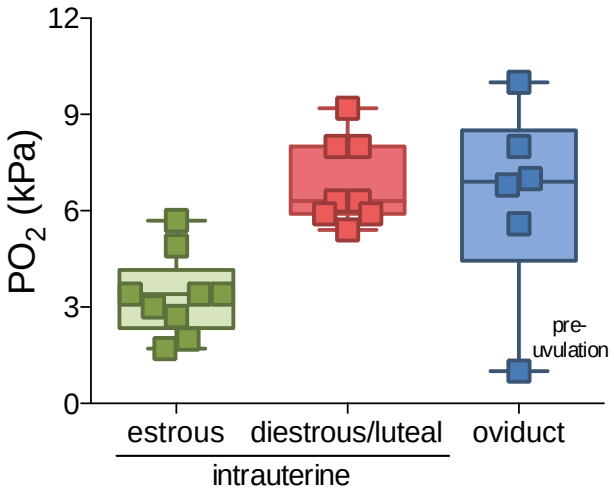

12
9
6
3
0
PO2 (kPa)
pre-
uvulation
estrous
diestrous/luteal
oviduct
intrauterine

Figure 18